# DIASTASE IN THE OPOSSUM (*Trichosurus Vulpecula*)

by

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In 1908 Wohlgemuth showed that the urine of man possessed diastatic activity, and he evolved a simple method for measuring this activity, Wohlgemuth's unit of diastase is defined as the number of cubic centimeters of 1 p.c. starch solution digested by 1 c.c. of urine, and the diastase index is the number of Wohlgemuth units per c.c. of urine. Starch is regarded as being digested or split when it reaches the point, determined titrimetrically under defined conditions, at which it no longer gives a blue colour with iodine. The test is usually carried out at pH 6–7, and it has been established that in man the urine normally contains from 7 to 33 and the blood from 3 to 10 Wohlgemuth units per c.c.

This measurement of diastatic activity, of course, does not define the type or types of amylase or diastase present. Lathan Crandall (1935) considers that there are at least two amylases (liquefying and saccharogenic) present in human blood: presumably both blood and urine (in so far as the kidneys are able to excrete these colloids) contain a mixture of all the amylases present in the organism, and this test affords a measure of their combined activity at the conditions of the experiment in degrading starch to a certain point. It is a convenient way of measuring this activity, and affords a basis for more detailed studies. The Wohlgemuth estimation has been employed largely in the work reported here, which is intended as a preliminary enquiry into the amyloclastic enzymes of the opossum. In the following studies "diastase" is used as a general term to cover the starch-splitting activities of unspecified amylases.

*Determination of the Normal Range of Diastase in the Urine and Plasma of the Opossum.*

As a preliminary step, four adult opossums were placed in separate urine-collecting cages with tinned floors and sieved drains. Twenty-four hour specimens of urine were collected in glass jars containing two drops of toluol. Samples of these were diluted with Sorensen's mixed buffer solution, and the pH values adjusted by means of $\frac{N}{10}$ NaOH or HCl to the various values shown in Table 1, B.D.H. Universal Indicator being used to approximately designate the pH. The Wohlgemuth titrations were then carried out in the usual manner.

The effect of adding NaCl to give a concentration of 0·4 p.c. in each tube was also tested.

The results of these tests, recorded as percentage activities, are set out in Table 1, the highest index obtained before the addition of NaCl being taken as 100 p.c.

TABLE 1.

*Activity of the Urinary Ferment at Various pH Values, and the Co-Activation Effect of Added NaCl.*

| pH. | Specimen. | Wohlgemuth Index. | Percentage Activity. | Wohlgemuth Index after added NaCl. | Percentage Activity. |
|---|---|---|---|---|---|
| 3–4 | 1 | 8 | 6·4 | 8 | 6·4 |
| | 2 | 10 | 10 | 10 | 10 |
| | 3 | 12 | 9·2 | 12 | 9·2 |
| | 4 | 8 | 9 | 9 | 10 |
| 5 | 1 | 20 | 16 | 30 | 24 |
| | 2 | 40 | 40 | 50 | 50 |
| | 3 | 45 | 30 | 50 | 37 |
| | 4 | 30 | 34 | 35 | 39 |
| 6·5 | 1 | 50 | 40 | 80 | 64 |
| | 2 | 57 | 57 | 90 | 90 |
| | 3 | 60 | 46 | 95 | 73 |
| | 4 | 55 | 62 | 80 | 91 |
| 7–8 | 1 | 125 | 100 | 140 | 112 |
| | 2 | 100 | 100 | 120 | 120 |
| | 3 | 130 | 100 | 170 | 131 |
| | 4 | 88 | 100 | 110 | 125 |
| 9–10 | 1 | 73 | 58 | 80 | 64 |
| | 2 | 60 | 60 | 75 | 75 |
| | 3 | 70 | 54 | 90 | 70 |
| | 4 | 60 | 68 | 70 | 79 |

It was concluded that the urine of the opossum showed diastatic activity, that the optimum pH for demonstrating this activity was 7–8, and that NaCl had a definite co-activating effect.

Subsequent estimations on urine, therefore, were all carried out after diluting with buffer and adjusting to this pH range, and a solution of starch in 0·9 p.c. NaCl used.

To determine the normal range of diastase in the urine of the opossum, 24-hour specimens were estimated daily, for periods of from 4 to 10 days, from a number of animals of all ages and both sexes, throughout one year. The volumes were noted, and the 24-hour output calculated, it being found that on account of oliguria, polyuria, etc., this figure afforded a much more reliable indication than the index itself. As a general rule the urine of the opossum on a predominantly eucalyptus diet tends to be alkaline, while on a bran-pollard-blood-meal-mash diet it tends to be acid, although there are individual exceptions to this. Where the 24-hour specimens were acid in reaction, they were allowed to stand one hour after adjustment of the pH, since it has been shown (see Haldane, 1930) that enzymes, inactivated

by an unfavourable pH, regain their activity to some extent on standing at optimum pH.

Animals were fed on bran-pollard-blood-meal-mash and eucalyptus leaves. In order to detect any variation due to age, sex, or season, the results were sorted into the groups shown in Table 2.

At the termination of the urinary observations on each animal, blood was obtained from the I.V.C. under ether anaesthesia. The citrated plasma was estimated after dilution with distilled water against saline starch.

TABLE 2.

*Urinary and Plasma Diastase in Normal Opossums.*

| | Animal. | 24-hour Urinary Output. Units. | | | Urinary Index. Units/c.c. | | | Blood Plasma. |
|---|---|---|---|---|---|---|---|---|
| | | Maximum. | Minimum. | Average. | Maximum. | Minimum. | Average. | Units/c.c. |
| Summer Adults | Male | 3,000 | 900 | 1,827 | 50 | 30 | 36 | 66 |
| | Male | — | — | 1,200 | — | — | 40 | 57 |
| | Female | 1,900 | 1,528 | 1,744 | 100 | 84 | 92 | 50 |
| | Female | 2,808 | 1,020 | 1,815 | 25 | 15 | 20 | 85 |
| Winter Adults | Male | 2,375 | 450 | 1,475 | 160 | 50 | 100 | — |
| | Male | 4,000 | 2,875 | 3,430 | 115 | 25 | 57 | 53 |
| | Male | 2,500 | 1,500 | 2,000 | 100 | 100 | 100 | 40 |
| | Female | 2,000 | 600 | 1,190 | 160 | 40 | 93 | 60 |
| | Female | 3,210 | 1,500 | 2,072 | 55 | 15 | 33 | 85 |
| Summer Young Animals | Male | 1,920 | 1,590 | 1,755 | 120 | 106 | 113 | — |
| | Female | 3,060 | 1,038 | 1,771 | 30 | 25 | 26 | 40 |
| | Female | 1,750 | 990 | 1,500 | 50 | 33 | 42 | 40 |
| Winter Young Animals | Male | 1,400 | 330 | 670 | 123 | 66 | 80 | — |
| | Male | 2,964 | 1,048 | 1,963 | 88 | 60 | 58 | 60 |
| | Female | 3,000 | 1,500 | 2,000 | 106 | 98 | 100 | 55 |
| | Female | 2,472 | 1,134 | 1,580 | 60 | 40 | 50 | 50 |

The results show that while plasma indices range from 40 to 85 units, the urinary indices are much more varied, ranging from 15 to 160 units. They were found to reflect, fairly closely, the concentration of the urine. The 24-hour output also varies considerably—from 330 to 4,000 units. The average values reveal no appreciable differences between ages, sexes, and seasons.

The possible influence of diet on the urinary diastase output was also investigated, by comparing the urinary output of animals on a pure eucalyptus leaf diet with that of the same animals on a bread and jam diet. It was found that the urinary indices were higher on a bread and jam diet, but the volume of urine excreted was reduced. The 24-hour urinary diastase output was within the normal range.

*Urine and Plasma Diastase in Lactating Opossums.*

Observations on lactating opossums were also carried out in the manner described above. The animals, which were selected as being in apparently good health, were divided into two groups: the first group consisted of animals with

small, unfurred pouch young, while the second group comprised animals with quarter-grown young on their backs, representing the declining stages of lactation. The figures found are set out in Table 3.

TABLE 3.

*Urinary and Plasma Diastase in Lactating Opossums.*

| | 24-hour Urinary Output. Units. | | | Urinary Index. Units/c.c. | | | Blood Plasma. |
|---|---|---|---|---|---|---|---|
| | Maximum. | Minimum. | Average. | Maximum. | Minimum. | Average. | Units/c.c. |
| Pouch Young | 6,528 | 1,028 | 2,961 | 257 | 133 | 216 | 50 |
| | 1,960 | 1,528 | 1,744 | 100 | 84 | 92 | 50 |
| | 12,150 | 3,000 | 5,860 | 250 | 100 | 230 | 58 |
| | 6,300 | 1,000 | 3,164 | 200 | 80 | 138 | 60 |
| Young on Back | 5,700 | 1,950 | 4,290 | 100 | 40 | 64 | 66 |
| | 9,900 | 920 | 4,250 | 200 | 8 | 70 | 66 |
| | 2,600 | 1,075 | 2,251 | 25 | 20 | 24 | 68 |
| | 17,500 | 2,640 | 6,700 | 100 | 15 | 51 | 57 |

It is evident that the output of urinary diastase is raised in both groups, whilst the plasma indices remain normal.

It has been noted in a previous paper (Anderson, 1937) that lactating opossums show an increase of reducing substances in the blood; the increased diastatic activity observed in lactation may be connected with the demand for the simpler reducing substances, formed by the breakdown of complex carbohydrates, for transference by the milk to the developing young.

The mammary glands themselves can excrete diastase; there is an $\alpha$-amylase present in cow's milk, and Manicatide, Brateseu and Popa (1935) recorded that human colostrum contained much more diastase than normal human milk, which in itself shows up to fifty times the concentration of diastase found in normal human serum. The normal and low serum diastase values found in human lactation may be accounted for by the activities, secretory or excretory, of the mammary gland, and in the opossum, which appears to maintain a normal renal diastase threshold during lactation, there is also the possibility that the increased diastase excretion in the urine reflects the activity of the mammary gland.

Direct evidence is lacking. In the tissue estimation experiments recorded below two lactating females, B and C, were examined. The post mortem amyloclastic activity of the liver, kidneys, spleen, pancreas, and stomach of each of these animals did not appear to differ from the rest of the series. Animal C showed a raised duodenal, intestinal and caecal amyloclastic content, but there was some evidence of enteritis. In any case these tissue estimations do not afford a true physiological picture.

*Amyloclastic Activity of the Gut and the Organs of the Opossum.*

The main source of the origin of diastase in man has been variously attributed to the liver, the pancreas, the salivary glands, and even to the glands of the stomach

—though the last-named source appears unlikely, since the stomach contents are too acid in health to permit the action of an amylase likely to influence significantly the Wohlgemuth titre of blood and urine carried out at pH 6–7.

To attack the problem of the source of urinary diastase in the opossum, the first step is obviously to discover what organs, tissues or secretions possess amyloclastic activity. A broad preliminary differentiation of the amylases found can then be achieved by finding the pH values at which they exhibit this activity—for instance, if the stomach can split starch at pH3 and not at pH9, while the caecum can split starch at pH9 and not at pH3, it may be assumed that the amylase of stomach is different from the amylase of caecum.

Since it is easier, although the results would be different, to investigate the diastase content of an organ after death than of its secretion during life, examinations of the tissues themselves were carried out. In the first place, attempts were made to estimate the Wohlgemuth titre of dilute alcohol, water, saline and 50 p.c. glycerol extracts of the tissues. Samples of fresh tissue were ground with quartz sand to break up the cells, and after standing and shaking with the extractive employed, the supernatant fluid, or the filtrate, was estimated by the usual technique. The results obtained were low and very variable. There was so much likelihood that the enzymes were incompletely extracted, were destroyed by dilution and standing, and were adsorbed on to the solid material, that this method was abandoned.

The method of Caldwell and Hildebrand (1935) was found to be satisfactory as applied to tissue determinations. The technique employed was as follows:

Animals were killed with ether, and the liver, spleen, kidneys, pancreas, stomach, upper intestine and caecum immediately removed and dissected free from fat, membranes, etc. The liver, spleen and kidneys were sliced, and washed free of blood by placing under a running tap for several seconds; and then dried by lightly pressing with blotting paper. The stomach, intestine, caecum, etc., were slit, and their contents washed out with running tap water—after drying with blotting paper, the mucous lining of these specimens was scraped off with a scalpel for estimation. All specimens were placed on ice as soon as possible.

When cold, each tissue was minced finely with scissors and thoroughly mixed. Representative samples, 1 gm. wet weight (0·1 gm. in the case of the pancreas) were taken of each specimen. These were ground to a homogeneous pulp with double-acid washed and ether-extracted sand in a mortar with 10 c.c. of extracting fluid. This was transferred qualitatively to an Erlenmeyer flask and 25 c.c. (100 c.c. in the case of the pancreas) of 2 p.c. soluble starch in 0·9 p.c. saline were added. The specimens were then brought to a temperature of 37°C. by rotating for a few minutes in a water bath, and then placed in an incubator at 37° for half-an-hour, when further action was stopped by immediately bringing to boil on a hot-plate. A control specimen, prepared as above, but boiled immediately after addition of starch to stop the action, was estimated with each analysis. The tissue material was separated from the starch solution by centrifugation, and the unchanged starch in aliquot volumes precipitated and weighed according to Caldwell and Hildebrand. By subtracting the weight of the tested precipitate from the weight of the control precipitate, the weight of starch transformed beyond the stage when it can be precipitated by 55 p.c. alcohol by 1 gm. wet weight of the tissue examined is obtained. For the purposes of this work, one gram of tissue exhibits one unit of amyloclastic activity when it transforms one mgm. of starch.

Different extracting fluids, e.g. alcohol, glycerine, electrolyte solution, extract different enzymes, or different forms of the same enzyme, from tissues. The extracting fluid chosen as being most likely to elute those enzymes that are estimated in the urine was a diluted Sorensen's buffer solution, with $\frac{N}{10}$ NaOH or $\frac{N}{10}$ HCl added to give 3 different pH values, viz.: pH 3, pH 7·5 and pH 9. It was found by test with B.D.H. universal indicator that these pH values remained the same after incubation with the various tissues and starch.

Results were as follows:

TABLE 4.

*The Amyloclastic Activity of Tissues of the Opossum at Three Different pH Values.*

| Tissue Examined. | Wet Weight of Tissue. gm. | Amyloclastic Activity. mgm/gm. pH 7–7·5 | pH 8·5–9 | pH 3–4 | Total Amyloclastic Activity. mgm. pH 7–7·5 | pH 8·5–9 | pH 3–4 |
|---|---|---|---|---|---|---|---|
| Liver A | 38·0 | 10·5 | — | — | 400 | — | — |
| Liver B | 42·0 | 31·4 | — | — | 1,319 | — | — |
| Liver C | 48·5 | 24·5 | 5·4 | 37·1 | 1,188 | 262 | 1,799 |
| Liver D | 47·0 | 46·2 | 50·6 | 56·0 | 2,171 | 2,378 | 2,632 |
| Liver E | 29·0 | 27·0 | 14·0 | 35·0 | 783 | 378 | 1,015 |
| Kidneys A | 11·0 | 55·7 | — | — | 613 | — | — |
| Kidneys B | 13·0 | 63·0 | — | — | 819 | — | — |
| Kidneys C | 11·6 | 85·9 | 56·7 | 45·1 | 996 | 668 | 523 |
| Kidneys D | 10·0 | 121·8 | 62·3 | 77·0 | 1,218 | 623 | 770 |
| Kidneys E | 8·5 | 77·0 | 55·2 | 58·4 | 654 | 469 | 475 |
| Spleen A | 2·2 | 6·1 | — | — | 13 | — | — |
| Spleen B | 4·0 | 0 | — | — | 0 | — | — |
| Spleen C | 3·2 | 0 | 0 | 55·0 | 0 | 0 | 176 |
| Spleen D | 4·0 | 0 | 0 | 165·2 | 0 | 0 | 660 |
| Spleen E | 1·6 | 13 | 0 | 125 | 20 | 0 | 200 |
| Pancreas A | 2·5 | 7,705 | — | — | 19,162 | — | — |
| Pancreas B | 1·9 | 11,760 | — | — | 22,344 | — | — |
| Pancreas C | 2·5 | 12,562 | 14,190 | 0 | 31,405 | 35,475 | 0 |
| Pancreas D | 3·56 | 13,060 | 15,230 | 0 | 46,493 | 54,218 | 0 |
| Pancreas E | 2·2 | 15,400 | 16,456 | 0 | 33,880 | 36,203 | 0 |
| Stomach A | 9·0 | 0 | — | — | 0 | — | — |
| Stomach B | 3·2 | 0 | — | — | 0 | — | — |
| Stomach C | 5·7 | 0 | 42 | 79 | 0 | 239 | 450 |
| Stomach D | 5·8 | 0 | 0 | 84 | 0 | 0 | 487 |
| Stomach E | 5·6 | 0 | 0 | 90 | 0 | 0 | 494 |
| Duodenum A | 17·0 | 83·8 | — | — | 1,425 | — | — |
| Duodenum B | 20·0 | 56·6 | — | — | 1,132 | — | — |
| Duodenum C | 16·0 | 282·8 | 277 | 122·5 | 4,525 | 4,432 | 1,958 |
| Duodenum D | 18·7 | 140·0 | 133 | 47 | 2,658 | 2,487 | 879 |
| Duodenum E | 17·4 | 78 | 86 | 30 | 1,357 | 1,496 | 522 |
| Intestine A | 15·6 | 91 | — | — | 1,429 | — | — |
| Intestine B | 15·9 | 115 | — | — | 1,828 | — | — |
| Intestine C | 17·4 | 289 | 258 | 225 | 5,028 | 4,289 | 3,915 |
| Intestine D | 15·0 | 90·3 | 73·5 | 40·6 | 1,354 | 1,102 | 609 |
| Intestine E | 12·6 | 101·5 | 113·4 | 23·1 | 1,279 | 1,428 | 29 |
| Caecum A | 6·5 | 0 | — | — | 0 | — | — |
| Caecum B | 3·5 | 7·7 | — | — | 27 | — | — |
| Caecum C | 7·2 | 38·4 | 5·6 | 32·4 | 276 | 544 | 233 |
| Caecum D | 9·8 | 7·7 | 14·7 | 0 | 75 | 144 | 0 |
| Caecum E | 7·6 | 0 | 0 | 0 | 0 | 0 | 0 |

The animals yielding the tissues for the above experiments were as follow:

*Animal A.* Female. Organs normal in appearance. Average 24-hour urinary diastase, 2,072 units (range, 3,210–1,500 units). Plasma, 85 units.

*Animal B.* Lactating female, ¼ grown young on back. Organs normal in appearance. Average 24-hour urinary diastase, 4,290 units (range, 5,700–1,950 units). Plasma, 59 units.

*Animal C.* Lactating female, ½ grown young on back. Spleen nodular in appearance and some inflammation in upper intestine. Other organs appeared normal. Average 24-hour urinary diastase, 6,700 units (range, 17,500–2,640 units). Plasma, 66 units.

*Animal D.* Young male. Duodenum inflamed and congested. Other organs normal in appearance. Average 24-hour urinary diastase, 2,400 units (range, 4,200–1,780 units). Plasma, 60 units.

*Animal E.* Young female. Organs normal in appearance. Average 24-hour urinary diastase, 1,770 units (range, 3,060–1,038 units). Plasma, 40 units.

In examining the above results, it is obvious that of the tissues examined, the pancreas shows the highest amyloclastic activity, and is responsible for about 87 p.c. of the sum total of the amyloclastic activity of the tissues examined—the well known rich source of diastase, salivary glands, not having been included. It must be remembered that estimations of the diastatic content of an organ obtained after slaughtering may bear no relation to the diastase forming ability of that organ. In the first place, we are measuring *in vitro* probably a different kind of diastatic activity to that which the organ carries out in life; and in the second place, since an organ which is capable of rapidly furnishing diastase may be capable of pouring it away as rapidly, it may show, at a given moment, a less diastatic content than an organ which neither makes nor loses it as rapidly.

The analyses recorded show that under the experimental conditions described, the pancreas has its highest activity at a definitely alkaline reaction, and shows no activity when the pH is 4–3. The stomach mucosa reveals a low activity at pH4–3 only, the duodenum has its optimum on the alkaline side, and the intestine somewhere about neutral; both the duodenum and intestine exhibit the same order of activity at pH values from 7 to 9, and show a definite diminution at acid reactions. The activity of the mucosa of the caecum, like that of the stomach, is apparently very slight—two specimens showed most activity at pH 8·5–9. The optimum for both the liver and the spleen appears to be pH 3–4, the liver showing the same order of activity up to pH7, diminishing rapidly at pH 8·5–9, while the spleen shows practically no activity other than at pH3. The maximum activity of the kidneys was at pH7–7·5.

It will be noted that both animals C and D, who had some inflammation of the gut mucosa, show comparatively raised values in the duodenum and caecum, and that the lactating animal C also showed raised values in the intestine.

The optimum pH ranges noted above become interesting when the reactions in the alimentary canal are considered. The diet of these animals was acid in reaction (pH3 downwards by the B.D.H. indicator); four varieties of gum leaves at different seasons gave an acid reaction on macerating with sand; while the mixed

mash was also acid. The saliva of the opossums was not tested, because of difficulties in collecting it: presumably both its reaction and diastatic content would vary with the food. The stomach contents and the bile, tested in 19 animals, were invariably acid (pH3 by B.D.H. indicator). The lower end of the small intestine showed a pH of 8, and the caecum pH 8·5–9. The upper end of the duodenum was between pH 4–6 as a general rule, when bile was obviously flowing, while the upper intestinal contents became progressively alkaline from this value towards the caecal end. In several cases, however, where bile did not appear to have freshly poured into the intestine, the reaction of the duodenum was pH 7–7·5. Estimations of bile, buffered and unbuffered and with and without NaCl as a coactivator, were made at pH ranges 3–9, and in no case was any diastatic activity exhibited by the Wohlgemuth method.

Such activity as the mucosae of stomach and caecum exhibited above was demonstrated at pHs of about 3 and 9 respectively, which is what would be expected on considering the reactions of their contents. But the reaction of the bile, which flows into the duodenum alongside the pancreatic juice, is definitely unfavourable to the amyloclastic activity of that juice, which is actually completely inhibited at pH 3–4. Similarly the enzymes of the duodenum, whose contents must on the evidence be usually acid, show their optimum activity on the alkaline side.

To test the vulnerability of the pancreatic enzyme, preparations of the pancreases from two animals were carried out at pH 3 in the manner previously described. These were allowed to stand for half-an-hour, and were then adjusted with $\frac{N}{10}$ NaOH to pH 8·5. After standing a further half hour, starch was added and they were incubated with control tests in the usual manner. Taking the control specimen at pH 8·5 to exhibit 100 p.c. activity, the controls at pH 3 showed no activity, while the test specimens, whose pH values had been re-adjusted, showed a loss of 34 p.c. and 40 p.c. activity respectively. Thus, standing at optimum pH for half-an-hour after previous exposure to unfavourable pH restores about 60–70 p.c. of the amyloclastic activity of the pancreas.

Higher values, of course, probably obtain in the gut itself, with more prolonged action and with the operation of coactivating factors, inhibitors of anti-enzymes, etc., in the intestinal secretions. When we remember also that the extracting fluids employed do not elute the same enzymes, or the enzymes in the same form, as are secreted by the various components of the digestive system, and that the pro-enzymes present may entirely escape detection due to absence of such converting factors as they would normally meet in the secretions of the organism, we realize that simple *in vitro* experiments such as are recorded above fall very short of giving a true indication of enzyme physiology as obtaining in the organism.

As far as the part contributed by the tissue amylases to the Wohlgemuth titre of the blood and urine is concerned, the analyses above give the following theoretical indications:

The stomach and spleen (slight activity at pH 3–4) and caecum (slight activity, mainly at pH 8·5–9) would be unlikely to have any effect. Possible con-

tributors would be the liver, kidneys, pancreas, duodenum, and small intestine, which all show diastatic activity in the pH range of the urine and blood estimations. Of these sources, though the pancreas is the richest, it has the smallest blood supply, and, in health, the blood would not absorb much enzyme from it. More absorption is likely to take place from the duodenum and upper intestine, whence the pancreatic amylase itself may also be absorbed.

Observations on the influence of the liver and of the pancreas on the blood and urine diastase titre of the opossum will be discussed in the following paper.

## SUMMARY.

1. The urine of the opossum exhibits its optimum diastatic activity at pH 7–8, and NaCl shows a co-activating effect.

2. The normal range of the 24-hour urinary diastase output of the opossum varies from 330 to 4,000 Wohlgemuth units, and averages about 1,750 units; the urinary indices range from 15 to 160 units/c.c., averaging about 65 units/c.c. The values found do not appear to be affected by age, sex, season or diet. The plasma indices range from 40 to 85 units/c.c., and average about 60 units/c.c.

3. The plasma diastase of opossums in both the early and declining stages of lactation is within the normal range: the 24-hour urinary diastase output is much raised, ranging from 920 to 17,500 units, and averaging about 4,000 units.

4. Post-mortem estimations of the amyloclastic activity of various tissues reveal widely different optimum pH values for exhibition of this activity: the connection of this with the physiology of the organism is discussed.

5. Of the tissues examined, the pancreas shows the greatest amyloclastic activity, but significant quantities were also found in the liver, kidneys, and upper intestine; and there is some evidence that increased amyloclastic activity may be exhibited by the intestine in enteritis.

6. From the evidence presented, inferences as to the possible contribution of each tissue to the starch-splitting activity of plasma and urine as estimated at pH 7–8 are drawn.

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# THE INFLUENCE OF THE LIVER AND THE PANCREAS ON THE BLOOD AND URINARY DIASTASE OF THE OPOSSUM

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In a preceding paper, estimations of the normal range of blood and urinary diastase and of the post-mortem amyloclastic activity of the tissues of the opossum were reported, and the possibilities of obtaining direct evidence as to the sources of the amylases in the blood and urine discussed.

The studies here described represent an attempt to establish by experimental ablation of two probable major sources of the blood and urinary diastase, whether the liver and the pancreas have any influence on the Wohlgemuth titre of the blood and urine.

*The Influence of the Pancreas on the Blood and Urinary Diastase of the Opossum.*

In the clinical laboratory, high diasturia has long been recognized as being associated with acute pancreatitis, trauma of the pancreas, etc., and is often taken as being diagnostic of that condition. When in other conditions, such as pneumonia and gastritis, a high diasturia or diastemia is noted, authors have assumed a coincidental involvement of the pancreas. In theory, the inflammatory condition causes obstruction of the duct or alters the permeability of the cells of the pancreas, so that its diastase passes straight into the blood, thence to be rapidly excreted to the urine.

In the opossum, the pancreas is a diffuse organ weighing about 3 grams, found in the usual situation in the meso-duodenum and extending to the spleen. The pancreatic duct joins the C.B.D. about a quarter of an inch from its opening into the duodenum: accessory ducts opening into the C.B.D. are common. In order to expose the pancreatic and upper part of the C.B. duct, it is necessary to divide the overlying head of the pancreas.

To test the question whether obstruction of the pancreatic duct causes an increase in the amounts of blood and urinary diastase in the opossum, the following experiments were carried out:

Animals were placed in urine-collecting cages, and their urinary diastase excretion observed for several days. They were then anaesthetized with ether, blood for estimation was taken from

the I.V.C., and two silk ligatures tied about an eighth of an inch apart, on the combined pancreatic and C.B. duct above its opening into the duodenum. The duct was then divided between the ligatures, the abdomen closed, and the animals returned to the cages where observations on their 24-hour output of urinary diastase were continued.

A summarized description of each experiment follows:

*Animal 1.* Large male on a diet of eucalyptus leaves and apples, observed five days in the urine-collecting cage. 24-hour urinary diastase excretion: maximum 1,920, minimum 1,200, average 1,425 units. Citrated blood plasma, 57 units/c.c. At operation, liver and other organs examined and appeared healthy. Combined pancreatic and C.B. duct ligated and divided after splitting of the overlying pancreas and consequent spilling of its secretion. Abdomen closed, animal returned to cage and observed for a further seven days. 24-hour urinary diastase excretion raised to 3,000–4,800 units during first four days, then decreased to 1,600 units on 7th day, when animal killed with ether, blood taken for estimation and abdominal contents examined. Blood plasma, normal (not icteric) in appearance: 35 units/c.c. O.E., liver healthy in appearance, G.B. nearly empty, bile flowing in duodenum, hence bile ducts not occluded. Large fibrotic calcified mass in region of head of pancreas, outlying portions of organ being pale, shrunken and transparent. Fat necrosis throughout abdominal cavity. No normal pancreatic tissue seen, and very evidently complete pancreatic achylia with almost complete pancreatic degeneration has been established.

*Animal 2.* Old lactating female, diet as in 1, observed five days. 24-hour urinary diastase excretion: maximum 1,960, minimum 1,530, average 1,830 units. Plasma, 50 units/c.c. At operation, a few small liver lesions noted, other organs healthy. Combined ducts ligated and divided without any trauma of the pancreas. Animal observed up to 6th day, when death occurred. 24-hourly urinary diastase excretion varied little, averaging 1,500 units and being 1,300 units on day of death. At P.M., G.B. hard and much distended. Evidence of much fat necrosis in the omentum and elsewhere. No pancreatic tissue macroscopically apparent, a fibrotic calcified mass representing the head of the organ.

As far as can be judged from the above two cases, the 24-hourly urinary diastase remains unaltered by complete pancreatic achylia with accompanying very extensive degeneration of the pancreas, although Animal 1 showed definitely raised values for some days, presumably because the pancreas had been directly ruptured at operation.

To test the effect of ligation and section of the pancreatic duct alone, without interference with the C.B.D., other animals had their main pancreatic duct exposed by dissecting off the pancreas in the region of the junction of the pancreatic duct with the C.B.D. The pancreatic duct was then divided between two silk ligatures.

Three animals were destroyed and examined 24 hours after the operation. In each case, the pancreas was pale, and distended throughout, with no congestion of its blood vessels. There was a good deal of necrosis evident in the fat of the mesenteries, and in two of the animals there was necrosis of the pancreas itself in the region of its dissection from the ducts. The plasma diatase before the operation in the three animals was 47, 61 and 61 units/c.c.: 24 hours afterwards, it was 40, 40 and 50 units/c.c. respectively. 24-hour urinary diastase before operation (average value) was 1,330, 1,660 and 1,840 units: for the 24 hours subsequent to the operation, the output was 420, 371 and 560 units respectively. It is evident that the post operative observation period is too short to permit significant observations to be made on the subsequent diastase absorption and excretion, although it is interesting to observe the early reaction of the pancreas itself before degeneration and atrophy occurs. The lowered plasma and urinary diastase at this early stage may be due simply to diminished vitality of the organism (the operation is much more severe than in the cases where the C.B. and pancreatic ducts were simply and quickly ligated and divided), or it may be because the normal absorption of the pancreatic enzyme from the gut is not

taking place, since the enzyme is no longer reaching the gut: and it is not being absorbed directly by the blood, the swollen cells still retaining their secretion.

Four other animals were observed for periods of 30 days up to 12 weeks after ligation and section of the pancreatic duct. Their 24-hourly urinary outputs were observed for 7 days before operation, and the blood obtained at operation estimated. In each case, the 24-hour urinary output did not vary by more than 500 units, and the plasma diastase was normal. After operation, in one case the 24-hour urinary diastase was much diminished for three days, when it returned to normal and remained at the pre-operation normal range for the rest of the period observed. In another case there was a transient rise up to 5,700 units on the day following the operation, then a return to and maintenance of the normal range.

In the third case, a rise to 8,000 units occurred on the second day, and then the excretion of normal values continued. It will be noted that neither of these two raised values exceed those observed in normal pregnancy (see preceding paper). In the remaining case, the normal range was maintained unchanged throughout the whole of the period observed subsequent to the operative procedure.

The animals were respectively destroyed at 1, 2, 2½ and 3 months after the operation. They were thin, and displayed an aversion from high carbohydrate foods, but otherwise seemed in surprisingly good health. The plasma diastase was below normal (25 and 30 units/c.c.) in two of the animals, and was 40 and 50 units/c.c. in the other two, compared to pre-operation values of 60 and 70 units/c.c. respectively. At P.M. two of the animals appeared to exhibit complete degeneration of the pancreas, with extensive fibrosis of its head; in the other two, accessory ducts were probably still functioning, as there seemed to be normal pancreatic tissue remaining in the outlying parts, although the head was fibrosed.[1]

It appears then that surgical trauma of the head of the pancreas, division of the pancreatic ducts and consequent atrophy of the pancreas, complete or partial, and pancreatic achylia, have no significant effect on the amount of diastase excreted by the urine in 24 hours. Since extensive fat necroses were also observed in the experimental animals, it may be deduced that a condition having some of the effects of acute pancreatitis has also obtained and this has not been reflected in the amount of urinary diastase.

Various workers have established that in the dog also ligation of the pancreatic ducts causes degeneration and atrophy of the pancreas: and Milla (1934) notes that in the dog the serum and urinary amylases, after a preliminary rise, return to normal in 10–15 days. If the pancreas normally contributes largely to the blood and urinary diastase, then it would be expected that this would be lowered both by pancreatic degeneration and by pancreatic achylia, when the

[1] Histological confirmation of the above-described changes was unfortunately not obtainable here: however, Dr. C. H. Kellaway very courteously examined two specimens of pancreas, one of which I had designated, on its macroscopic appearance, as being "completely degenerated", and the other as being "extensively degenerated", and very kindly furnished me with the following microscopal reports:

*No. 1.* Extensive atrophy of secreting tissues of the pancreas. The section shows much duct tissue, and some islet cells though these are not as numerous as would be anticipated from their frequency in normal (opossum pancreas) sections. The remainder of the organ is fibrous tissue with no external secretion tissue at all.

*No. 2.* Here you seem to have tied an accessory duct: about half the section shows normal pancreatic tissue. There is a dilated duct in the section and there are considerable areas of fibrosis in the section which nowhere shows the appearances characteristic of pancreatic atrophy as seen in Section 1.

enzyme cannot be absorbed from the intestine: and actually some lowering of the plasma diastase was observed in the above experiments—the return to normal values may be evidence of increased compensatory secretion by the intestine.

*The Effect of the Liver on the Blood and Urinary Diastase of the Opossum.*

Somogyi (1936), Bartlett (1937) and others have noted that low blood amylase levels accompany liver dysfunction in man, and it has been held that the blood amylase is derived largely from the liver. In the opossum (see preceding paper) the liver was observed to exhibit considerable diastatic activity and if, in life, its amylases are significantly absorbed by the blood, then removal of the liver, cutting it off from the general circulation, or destroying it, would be expected to lower the Wohlgemuth titre of the blood and urine. On the other hand, lesions, inflammations, etc., might increase its diastatic activity or alter the permeability of its cells, and hence raise the blood and urinary diastase.

In considering the experimental trial of this hypothesis, it is obvious that neither removal of the liver nor tying off of its circulation would yield practical information, on account of the short survival time to be expected. Effects found would be attributable not to the liver alone but to the consequent shocking and dysfunction of the entire organism. But if by any means it were possible to cause necrosis, degeneration, atrophy, etc., so that sufficiently large amounts of liver tissue be thrown out of action, then observed effects on the blood and urinary diastase titres would be information.

It is known that numerous substances, including copper salts, act as poisons to liver cells and lead to cirrhosis and necrosis of the organ; and an attempt was made to secure the result by portal injection of 0·25 p.c. $CuSO_4$, but without effect.

It appeared, therefore, that sufficient destruction of liver tissue for the purposes of this experiment could not readily be procured by $CuSO_4$ injections, and the method was abandoned.

The method of Cameron and Mayes (1930) was then tried, and the necessary liver degeneration produced by ligation of the hepatic artery. In the opossum, the *a. hepatica propria* is a very small vessel ascending between the layers of the hepato-duodenal ligament, dividing into a variable number of branches to the various lobes, gall-bladder, gastro-duodenal artery, etc. Occasionally collateral branches may arise near the origin of the R. gastric artery and ascend to the *porta hepatis*. In the experiments conducted, the hepatic artery and such collateral supply as could be found, was ligated and divided above the origin of the gastro-duodenal artery.

The experiments and their results are summarized as follows:

*Animal A.* Adult male, average 24-hour urinary diastase (observed for a fortnight) 1,500 units: Plasma (obtained at operation) 80 units/*c.c.* At operation, all organs appeared healthy. Hepatic artery tied and divided, animal returned to cage, and 24-hour urine diastase observed for three weeks, when animal again examined under ether. Th average post operative 24-hour urine

diastase excretion was 600 units, the values having declined from a maximum of 1,500 units on the third day subsequent to the operation. Plasma diastase, 30 units/c.c. O.E. the liver was found to exhibit extensive necrosis.

*Animal B.* Large male, average 24-hour urinary diastase, 5,600 units. Plasma, 150 units/c.c. At operation, all organs normal except pancreas, which appeared inflamed. Hepatic artery tied and divided. Diastase excretion observed for subsequent three weeks: average 24-hour urine diastase 588 units. Animal examined under ether: plasma 26 units/c.c. O.E. the kidneys, spleen and pancreas appeared normal. Massive necrosis of left lobes of liver, small middle lobe unaffected, large abscess on right lobe. (See accompanying photograph.)

**Fig. 1.** Liver from Opossum B, showing damage caused by ligation and division of hepatic artery.

*Animal C.* Old lactating female, average 24-hour urinary diastase excretion, 4,450 units. Plasma, 60 units/c.c. At operating, liver showed some nodularity of left lateral lobe, other organs normal. Main stem of hepatic artery tied off, and urinary diastase excretion observed for four weeks. After the sixth day, it rose to very high levels, which were maintained for ten days and then the excretion was at pre-operative ranges. Maximum value in second week, 21,000 units: average value in fourth week 3,800 units. Animal examined under ether: plasma, 60 units. O.E. a small abscess found in left lobe of liver, which was cleaned and swabbed; rest of liver presented a fairly normal appearance. A network of arterial branches supplying the liver was found, also a branch from the gastro-duodenal artery: hence the liver had not been deprived of arterial blood. All vessels found were tied and divided, and the animal returned for further observation. It survived seven days, during which its 24-hour urinary diastase excretion averaged 350 units, being only 60 units on the day preceding death. Plasma not obtained for estimation. At P.M., massive necrosis involving all the lobes of the liver was observed; no normal tissue macroscopically apparent.

*Animal D.* Adult female, non-lactating. Average 24-hour urine diastase, 1,950 units. Plasma, 85 units/c.c. At operation, both liver and pancreas appeared normal: hepatic artery tied and cut, and animal observed for two weeks. 24-hour urine diastase excretion rose to 6,000

units on fourth day, then declined rapidly. In the second week it averaged 400 units, being 30 units on last day. Animal examined under ether: plasma (very icteric in appearance) 15 units/c.c. O.E. extensive fat necrosis throughout abdomen. Spleen and kidneys appeared normal. The pancreas was enlarged and calcified. The liver was very enlarged, mottled, necrosed and contained several large abscesses—no normal tissue seen.

Tests for urobilin and urobilinogen were carried out daily on the urines of the above animals, and in no case were they positive.

It appears from the above results that extensive necrosis of the liver leads to a pronounced diminution in excretion of the urinary diastase—values from $\frac{1}{3}$ to $\frac{1}{10}$ of the normal output being observed. The plasma diastase also falls below normal values. The transient raised values observed were apparently due to inflammatory reactions, and in case D may have been due to a coincidental pancreatic lesion; here the subsequent degeneration of the pancreas is excluded by the previous experiments as a factor in the lowering of diastase values.

It is concluded that the liver normally contributes largely to the amount of diastase found in the blood and urine of the opossum.

## SUMMARY.

1. Ligation and section of the combined pancreatic and common bile duct, and of the main pancreatic duct itself, was performed on the opossum. Subsequently the pancreas was found to be extensively atrophied. No significant alteration of the diastase content of the plasma and urine resulted. Transient rises, not exceeding the values found in normal lactation, were sometimes observed in the 24-hour urinary diastase immediately subsequent to operation, and appeared to be due to the surgical interference with the pancreas itself.

2. Direct injection of 25 mgm. of copper as $CuSO_4$ into the hepatic vein did not cause very extensive liver damage.

3. Extensive liver damage produced by ligation of the hepatic artery caused a pronounced diminution of the urinary and plasma diastase.

4. It is concluded that normally in the opossum the liver is an important source of the blood and urinary diastase.

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# A NOTE ON THE ESTIMATION OF REDUCED GLUTATHIONE

by

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*(Submitted for publication 12th March, 1938.)*

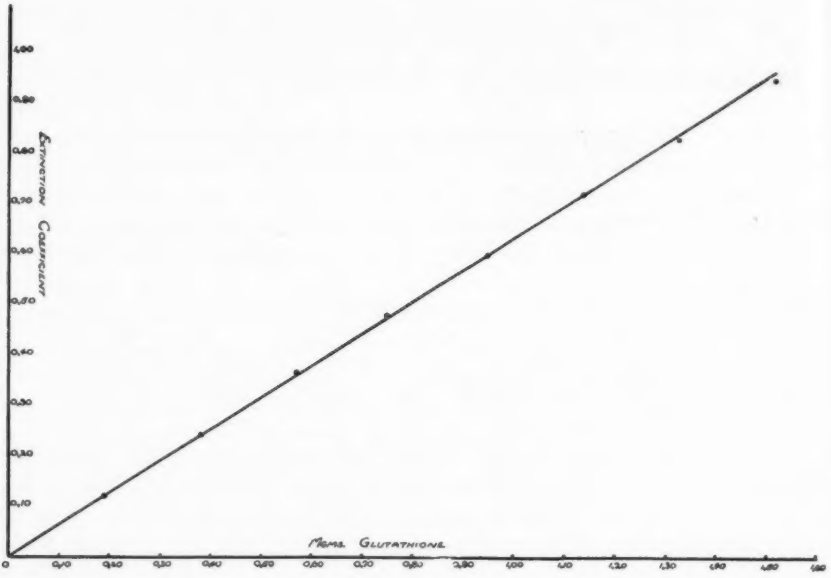
In some recent investigations we had occasion to estimate the reduced glutathione (G.S.H.) content of rabbits' liver. The most satisfactory method appeared to be that of Mason, but unfortunately its application to the particular material with which we were dealing proved somewhat unsatisfactory. In Mason's (1930) method a tungstic acid, protein-free filtrate is prepared from the tissue and the G.S.H. oxidized by means of N/100 potassium ferricyanide. The resultant ferrocyanide is converted into Prussian blue by the addition of Folin's ferric sulphate reagent and determined by colorimetric comparison with a standard solution of cysteine that has been treated in a similar fashion.

At the outset it is obvious that if the G.S.H. or cysteine is to be completely oxidized, a slight excess of ferricyanide must be added, but the yellow colour of this in the blue solution renders colorimetric comparison extremely difficult. To some extent this difficulty can be overcome by the addition of a few drops of ferricyanide to either the standard or the unknown solution, but on the whole this is an unsatisfactory procedure. A further complication arises when one is dealing with liver tissue, particularly from rabbits. Here, owing to the presence of varying amounts of glycogen, the protein-free filtrate possesses a distinct opalescence, thus rendering accurate titration with ferricyanide impossible. By means of the Zeiss Pulfrich photometer supplied through the generosity of Dr. Rowden White we have been able to overcome these difficulties. Our procedure has been to follow Mason's method up to the stage where ferrocyanide is converted into Prussian blue. The extinction coefficient of this coloured compound is then determined and, by reference to a calibration chart, the actual concentration of G.S.H. determined.

In Chart No. 1 the relationship between G.S.H. concentration and extinction coefficient for the particular filter that we are using is shown. A sample of the tripeptide prepared according to Pirie's method (1930) was supplied to one of us (A.B.C.) by Dr. King of the National Institute of Medical Research, London. A careful titration with ferricyanide indicated a G.S.H. content of 96·8 p.c.

In order to obtain the information shown in Chart 1, a solution of G.S.H. in 0·1 N HCl was prepared so that 1 cubic centimetre contained 0·76 milligrams of G.S.H. Such a solution will

remain stable for three months if kept in the ice chest. In estimating the extinction coefficient, the required amount of G.S.H. solution was placed in a 100 cubic centimetre volumetric flask and neutralized with 0·1 N NaOH. In order to bring the pH to 5·9, the phosphate buffer recommended by Mason was next added and the solution titrated to a definite yellow colour with N/10 potassium ferricyanide. The ferric sulphate reagent was then added and ten minutes allowed for the blue colour to develop.




After making up to volume, the extinction coefficient was determined with the filter designated by Carl-Zeiss as S72. The dominant wave length of the light transmitted by this filter is 720$\mu\mu$. This filter was chosen because it gave a sufficiently high absorption to ensure adequate accuracy and the light transmitted was ample to permit of easy reading. The approximately monochromatic nature of this filter eliminated interference due to the yellow colour of excess ferricyanide. As a control of the effect of excess ferricyanide on the light absorption, with the S72 filter a solution was made up in the same manner as the standard solution except that the G.S.H. was omitted. Sufficient ferricyanide was added to give an intensity of yellow colour equal to that usually present in actual estimations of G.S.H. Measurements of such control solutions gave values low enough to be within the personal experimental error in reading. In Chart I the extinction coefficients for the following proportionate amounts of G.S.H. are shown: 0·19 mg., 0·38 mg., 0·57 mg., 0·76 mg., 0·95 mg., 1·14 mg., 1·33 mg., 1·52 mg. In all instances the averages of at least six estimations were taken.

*Recovery of Added G.S.H.*

A rabbit was killed by a blow on the head and bled out from the carotid arteries. The liver was removed and finely minced. Ten grams of minced liver were placed in a mortar and thoroughly ground up with 70 c.c. of distilled water. Next, with constant stirring, 10 c.c. of 2/3 N

$H_2SO_4$ were slowly added and finally 10 c.c. of 10 p.c. Na tungstate. After allowing to stand for 30 minutes the mixture was filtered. Ten cubic centimetres of this protein-free filtrate were transferred to a 100 c.c. volumetric flask and the pH adjusted to 5·9 by the addition of phosphate buffer. The solution usually possessed a distinct opalescence and was titrated to a definite yellow colour with N/100 potassium ferricyanide. The Prussian blue colour was developed in the usual manner and the G.S.H. content obtained from the extinction coefficient. In this particular case it was found that 10 c.c. of protein-free liver extract contained 0·39 mg. G.S.H. Five 10 c.c. quantities of this extract were taken and progressively increasing amounts of G.S.H. added to each sample. The extracts were then treated in the usual manner for the determination of G.S.H. The results are shown in Table 1.

TABLE 1.

*Recovery of Added G.S.H. to Liver Extract.*

| Extract. | G.S.H. added. | G.S.H. found. | Theoretical. |
|---|---|---|---|
| 10 c.c. | Nil | 0·39 mg. | — |
| 10 c.c. | 0·2 mg. | 0·58 „ | 0·59 mg. |
| 10 c.c. | 0·4 „ | 0·79 „ | 0·79 „ |
| 10 c.c. | 0·6 „ | 0·98 „ | 0·99 „ |
| 10 c.c. | 0·8 „ | 1·20 „ | 1·19 „ |
| 10 c.c. | 1·0 „ | 1·39 „ | 1·39 „ |

*The Question of Opalescence.*

As previously mentioned, opalescent solutions are encountered with extracts from liver tissue, and accurate titration with ferricyanide is practically impossible. With such solutions our procedure has been to use an opalescent solution as a control and to titrate the unknown to a definite yellow colour. The question that arose was whether any appreciable light absorption was caused through the opalescence, in which case our deductions from the observed extinction coefficients would be fallacious. A large number of opalescent solutions were examined, purely from the standpoint of light absorption. The most markedly opalescent that was encountered gave a light absorption corresponding to an amount of G.S.H. less than 0·01 mg. For practical purposes this is negligible.

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# THE EFFECT OF HISTIDINE ON THE HISTAMINE CONTENT OF THE LUNGS AND ON HISTAMINE REACTIONS

by

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*(Submitted for publication 9th January, 1938.)*

In the experiments reported in this paper it was sought to ascertain whether the injection of histidine into a guinea-pig causes an increase in the histamine content of the lung and what influence histidine may exert on histamine, peptone and acetylcholine reactions.

Recent observations on the decarboxylation of histidine have strengthened the conception that the histamine found in the organs is derived from histidine. Histidine is decarboxylated by an enzyme (histidase) present in several organs (Edlbacher, 1926; Holtz, 1937; Holtz and Heise, 1937a) and by various naturally occurring substances, like glutathione, cystein and ascorbic acid in the presence of oxygen (Holtz and Heise, 1937a). The final proof that the histamine present in the organs results from the decarboxylation of histidine appeared to have been reached by the observation that the injection of histidine into guinea-pigs doubled the histamine content of their lungs. Bloch and Pinösch (1936) found that the histamine content of the guinea-pig's lung, which in their experiments varied between 15 and 25γ per gram tissue, increased to between 30 and 45γ five hours after the subcutaneous injection of histidine. It seemed desirable to repeat these experiments since the content of histamine in the guina-pig's lung is subject to individual variation so great that in one instance at least it led to erroneous conclusions in regard to the influence of different conditions upon the histamine content of organs. Watanabe (1930–31) and Hosoya and Watanabe (1931) in different allergic conditions, described changes in the histamine content of the lung and liver, which were clearly attributable to individual variation (Bartosch, Feldberg and Nagel, 1932; Daly, Peat and Schild, 1935). The histamine content per gram lung varied in Watanabe's experiments on guinea-pigs between 1 and 94γ, in those of Daly, Peat and Schild between 2 and 40γ, and in experiments carried out

[1] Seconded from Department of Human Physiology and Pharmacology, University of Adelaide, and sustained by a grant from the Scientific Research Endowment Fund of the Council for Scientific and Industrial Research.

in this Institute on 26 guinea-pigs in which extracts of the lungs were made with Tyrode solution, between 7 and 88γ (average 29γ). The range of individual variation is therefore large enough to cover all the values obtained by Bloch and Pinösch in normal guinea-pigs, as well as in those treated with histidine.

Edlbacher, Tucker and Baur (1937) found that the contraction of the isolated guinea-pig's gut to histamine is abolished by the presence of histidine in moderately high concentrations. It seemed important to determine whether histidine exerts a similar effect upon contractions produced by other drugs.

## METHODS.

For the examination of the effect of histidine on the histamine content of the lungs, guinea-pigs weighing from 310 to 715 gm. were used. Histidine in an amount of 0·1 gm. per 100 gm. body weight was injected subcutaneously or intraperitoneally. We used a 4 p.c. solution of histidine (Larostidin, Hoffmann-la Roche, Basel), which was found to be acid to litmus. In several experiments the solution was neutralized with NaOH before injection. Five hours after the injection the guinea-pigs were killed by a blow on the head, and the right and left lungs were removed and extracted separately in Tyrode solution. The method of extraction has been described by Feldberg and Kellaway (1937). In preliminary experiments we extracted one lung with Tyrode solution and the other with methyl alcohol after grinding the tissue with quartz powder. We invariably found, whether we were dealing with lungs of control guinea-pigs or of animals which had been injected with histidine, that the histamine equivalent obtained for the lung extracted by methyl alcohol was much smaller than that extracted with Tyrode solution, indicating a loss of histamine in the process of extraction by alcohol.

In order to reduce the error of individual variation in the histamine content of the lungs, we have in some cases extracted the lungs of two or three guinea-pigs together, extracting the left and right lungs separately. In each case control experiments on guinea-pigs which were not injected with histidine were carried out *on the same day*. The lung extracts were assayed on a piece of guinea-pig's jejunum suspended in a 3 c.c. bath of Tyrode solution against histamine dihydrochloride (Hoffmann-la Roche, Basel) and the histamine content of the lung is expressed not as base but as dihydrochloride.

To examine the effect of histidine on the contraction of the guinea-pig's gut produced by different drugs (histamine, histamine-free peptone (Peptone Berner) and acetylcholine) a piece of the upper portion of the jejunum was suspended in a 3 c.c. bath of Tyrode solution at 35° C. The histidine (0·2–0·4 c.c. of 4 p.c. solution) was added to the bath and the effects of different drugs were examined in its presence sixty seconds later, or the histidine was added to the bath when contraction of the gut had already been produced by the various drugs. The results were

the same, whether neutralized or the acid histidine solution was used. The effect of histidine on the depressor action of histamine in the cat was studied under chloralose anaesthesia after cutting both vagi.

## RESULTS.

*Histamine Equivalent of Guinea-pig's Lungs.*

The histamine content of the guinea-pig's lungs varies normally within wide limits. In the seventeen animals of Table 1 the histamine content per gram lung varied from 3·3 to 50γ (average 25·9γ). In another series of twelve guinea-pigs (Table 2) 1 gm. per kg. of histidine were injected; in the first eight animals the histidine was injected subcutaneously and the last four intraperitoneally. The histamine equivalent per gram lung in these animals varied between 2·8 and 40γ (average 25·6γ). These results show that the lung histamine of guinea-pigs treated with histidine lies in the normal range.

TABLE 1.

*Histamine Equivalent of Guinea-pig's Lungs.*

| Weight of Guinea-pig in grams. | Histamine equivalent in γ per gm. lung. Left lung. | Right lung. |
|---|---|---|
| 410, 530, 710 | 30 | 40 |
| 390, 560, 700 | 50 | 40 |
| 310, 345 | 25 | 20 |
| 755, 620 | 20 | 20 |
| 720 | 12·5 | 12·5 |
| 320 | 22 | 22 |
| 400 | 21 | 15 |
| 395 | — | 17·5 |
| 405 | — | 30 |
| 390 | — | 3·3 |
| 680 | — | 12·5 |

*The Effect of Histidine on the Reactions of Histamine, Peptone and Acetylcholine.*

*Guinea-pig's Jejunum.* The addition of histidine to the bath caused slight relaxation of tone and inhibition of spontaneous movements of the gut. When the histidine was left in contact with the muscle for a minute and then washed out, a slow contraction sometimes set in after about ten seconds. This after-contraction was absent in preparations which had a low sensitivity to histamine; on the other hand the after-contraction was strongest in preparations which were particularly sensitive to histamine. In Fig. 1 after-contractions of moderate size are seen.

In confirmation of the results of Edlbacher, Tucker and Baur we found that in the presence of histidine the effect of histamine was diminished or abolished (Fig. 1 G). We found that 8 mgm. of histidine were usually required to prevent the contraction of 0·04–0·1γ of histamine. If larger doses of histidine were used more histamine could be rendered ineffective. When the histidine was washed out the gut immediately regained its original sensitivity to histamine, and the inhibitory effect of histidine could be demonstrated repeatedly on the same piece of gut. When the gut was contracted by histamine and histidine added to the bath without washing out immediate relaxation set in.

TABLE 2.

*Histamine Equivalent of Guinea-pig's Lungs after Histidine.*

| Weight of Guinea-pig in grams. | Histamine equivalent in γ per gm. lung. Left lung. | Right lung. |
|---|---|---|
| 440<br>590<br>630 | 25 | 30 |
| 415<br>565<br>715 | 45 | 40 |
| 400 | 17·5 | 15 |
| 390 | 7·5 | — |
| 405 | — | 40 |
| 400 | — | 2·8 |
| 405 | — | 10 |
| 395 | — | 20 |

The inhibitory effect of histidine is not specific for the histamine contraction. We found that contractions of the gut produced by peptone and by acetylcholine were also prevented or abolished by histidine. Peptone and acetylcholine in doses which normally produced strong contractions were ineffective in the presence of sufficient histidine, or if histidine was added to the bath during contraction, immediate relaxation took place. The inhibitory effect of histidine on the acetylcholine contraction was not quite as strong as that on the histamine contraction. When equally strong contractions were subjected to the same concentration of histidine, the histamine contraction was inhibited to a greater extent than the acetylcholine contraction. This is illustrated by the experiment of Fig. 1. The contractions produced by 0·1γ of acetylcholine (at A) and by 0·1γ of histamine (at E) were about equally strong. In the presence of histidine the acetylcholine contraction was greatly reduced (at C), whereas the histamine contraction was completely prevented (at G). The difference in the inhibitory effects on the two drugs is not great, since—when the concentration of histidine was slightly reduced—the effect of 0·1γ of histamine was not completely prevented.

*Cat's Blood Pressure.* The depressor action of histamine was not influenced by histidine whether this was injected either simultaneously or before the injection of histamine. For instance in a cat under chloralose the intravenous injection of 1$\gamma$ of histamine caused a fall in arterial blood pressure of 16 mm. Hg. The same amount of histamine together with 0·5 c.c. of a 4 p.c. solution of histidine injected 4½ minutes later, produced a fall of 18 mm. Hg. The cat gradually became more sensitive to histamine and some ten minutes later 1$\gamma$ caused a fall of 28 mm. Hg. The injection of 3 c.c. of a 4 p.c. solution of histidine now caused a fall of 42 mm. Hg. When the blood pressure had recovered, two minutes later, 1$\gamma$ of histamine produced a fall of 26 mm. Hg.

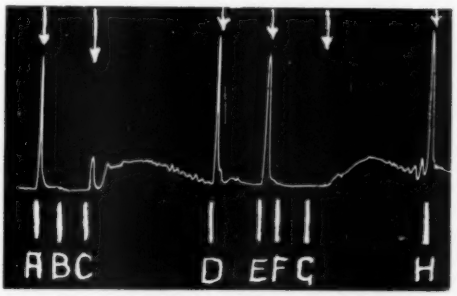


**Fig. 1.** Reactions of the isolated jejunum of the guinea-pig. At A, C and D, 0·1 $\gamma$ of acetylcholine; at E, G and H, 0·1 $\gamma$ of histamine; at B and F, 16 mg. of histidine were added to the bath. At the arrows ( ↓ ) the Tyrode solution was changed.

## DISCUSSION.

We have not been able to confirm the finding of Bloch and Pinösch (1937) that the injection of histidine into guinea-pigs increased the histamine content of the lungs. We found that the individual variations in the lung histamine of guinea-pigs are so great as to embrace not only the normal values obtained by Bloch and Pinösch, but also the values which they obtained in guinea-pigs injected with histidine. The general conception, however, that the histamine found in animal organs is derived from histidine is unaffected by our results though they do not supply evidence in its favour.

The inhibitory action which histidine exerts upon the contraction produced by histamine on the isolated jejunum is not a specific histidine-histamine antagonism. The contractions produced by peptone—and by acetylcholine, are with trivial quantitative differences, equally well inhibited by histidine. Histidine cannot therefore be used, as appears to be the case with arginine (Rothlin 1937), to differentiate between the contraction produced by histamine and that caused by other

drugs. Furthermore histidine has no inhibitory effect on the depressor action of histamine in the cat, and it is therefore uncertain how far it would produce such an effect on smooth muscle through which blood was normally circulating.

## CONCLUSION.

1. The injection of histidine does not produce changes in the histamine content of the lungs.

2. Histidine inhibits the contraction produced by histamine, peptone and by acetylcholine on the isolated guinea-pig's jejunum, but does not influence the depressor action of histamine in the cat.

## ACKNOWLEDGMENT.

I wish to thank Dr. W. Feldberg for his suggestion and help in this work.

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# THE EXCITABLE CORTEX IN THE MONOTREMATA

by

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*(Submitted for publication 3rd May, 1938.)*

## INTRODUCTION.

A survey of the cortical lamination pattern in *Ornithorhynchus* and *Tachyglossus* raised the problem: which, if any, of the areas determined histologically would exhibit motor functions? The experiments to be described were designed primarily for this specific purpose only, but their prosecution raised a number of allied problems each presenting the prospect of a lengthy investigation. It was considered advisable, therefore, to restrict this communication to the immediate purpose in hand—the simple demarcation of the excitable cortex—and to defer consideration of the related prospects for further investigation.

Experimental explorations of the cerebral cortex in the Monotremata are few, and the results inconclusive. Martin (1898) stimulated the forebrain in two specimens of *Ornithorhynchus* using faradic excitation under ether anaesthesia. He found the frontal two-thirds or so of the hemisphere diffusely excitable, but the movements obtained—of the forelimb, head and eyelids—were poorly localized and identified only after laborious search. In this department Professor Burkitt and Dr. G. E. Phillips have stimulated the cortex in several specimens of *Tachyglossus* but they were unable to obtain any satisfactory results. They employed galvanic stimulation under "dial" anaesthesia; it has been found since that "dial" abolishes all cortical movements in echidna. With Professor Burkitt's assistance the experiments on *Tachyglossus* were repeated. Bipolar faradic stimulation under ether anaesthesia provided a series of results sufficiently constant to warrant confidence in their accuracy. Two specimens of *Ornithorhynchus* were subjected to similar exploration with results comparable with those of Martin's. Later, with the assistance of Dr. Phillips, the technique was improved by the substitution of a thyratron tube as a source of excitation for some specimens of *Tachyglossus*. This later series was designed to explore some of the possibilities raised by the previous experiments, but insofar as they were concerned with cortical excitation they confirmed the earlier results.

I must express my thanks to Professor Burkitt and Dr. Phillips for their kindness in making available the results of their own experiments, and for their assistance with the present series.

## MATERIALS AND METHODS.

*Ornithorhynchus.* Only two living specimens were available. In each both hemispheres were explored with the bipolar faradic electrode under ether anaesthesia. The best results were obtained when the coils were separated to about 100–110 mm. These animals are very responsive to ether and anaesthesia may be prolonged under light administration on an open mask as required.

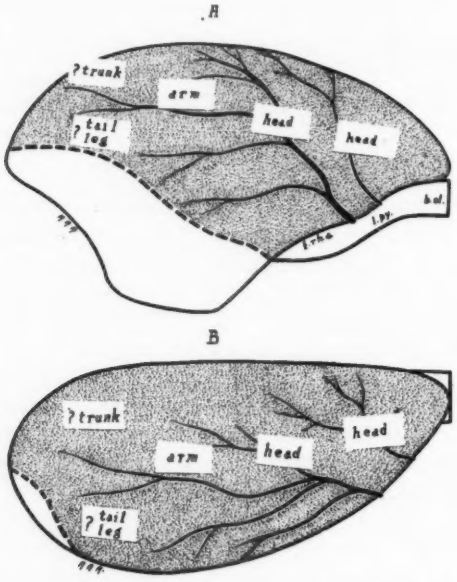


**Figure 1.** Total distribution of the excitable cortex in *Ornithorhynchus*, A from the lateral aspect, B from the dorsal aspect, × 2½. Note the large cerebral veins.

* ABBREVIATIONS EMPLOYED IN THE FIGURES.

| | |
|---|---|
| b.ol. | olfactory bulb. |
| f.ps. | pseudo-sylvian fissure. |
| f.rh.a. | anterior rhinal fissure. |
| f.rh.p. | posterior rhinal fissure. |
| l.py. | pyriform lobe. |

Letters of the Greek alphabet indicate sulci which were so entitled by Elliot Smith in his original description.
Roman numerals indicate specific cell laminae in the cerebral cortex.

As Martin has pointed out, the absence of sulci makes localization difficult and recourse was made to his expedient of employing the large cerebral veins for this purpose. The areas found responsive were compared with the cortical lamination pattern previously determined and their structure was checked by further histological examination.

* These abbreviations are employed in all figures.

*Tachyglossus.* In all eight specimens have been explored: five with the bipolar faradic electrode and three with the unipolar thyratron tube electrode. Of the former five, in two cases, and of the latter three, in one case, both hemispheres were examined, making a total of eleven hemispheres. In one of the latter series, however, "dial" anaesthesia was used as a control and there was no response to stimulation. Discarding this, ten hemispheres gave positive findings. Apart from the single control, "open" ether was employed in all cases and proved quite satisfactory. The minimum effective strength of the stimulating current was very constant from animal to animal: separation of the coils by 110 mm. in the faradic series, or an output of 250 mv.

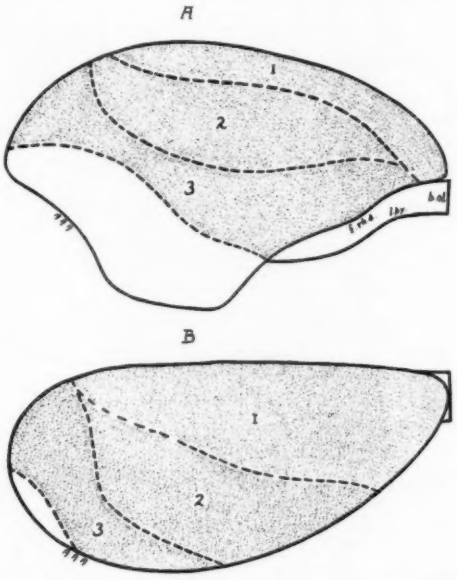


**Figure 2.** Graphic reconstruction of the right hemisphere in *Ornithorhynchus*, A from the lateral aspect, B from the dorsal aspect, to illustrate the distribution of the three cortical modifications concerned with motor function, × 2½.

at a frequency of approximately 40 cycles in the thyratron tube series, proved adequate in practically every case. In all the animals the hemispheres were explored as thoroughly as possible; in a few cases the olfactory bulbs were stimulated as well. After most experiments the animal was killed immediately, the brain exposed and the sulcal pattern carefully mapped. This is essential since Burkitt (1934) has shown that the sulci are subject to a considerable degree of variation. Sulcus *a* alone was found to be reasonably constant. In one animal, before sacrifice, the spinal cord was exposed and completely divided through the twelfth thoracic segment to determine whether or not movements of the hindlimbs and tail depended upon tracts traversing the cord. In another animal that part of the excitable cortex which controlled movements of the hindlimbs and tail was destroyed by applying Dusser de Barenne's laminar coagulator for five seconds at a temperature of 80 degrees centigrade in an attempt to obtain a Marchi degeneration of the

descending pathways. In many of the animals, in addition to the previous cortical survey, blocks of cortex were excised at right angles to sulcus α, sectioned and stained with cresyl-violet to check the original histological findings.

## RESULTS.

*Ornithorhynchus.* The results in all four hemispheres coincided with those of Martin to the extent that practically the whole of the dorso-lateral surface of the hemisphere was found to be diffusely excitable; only the postero-lateral corner did not respond (Fig. 1). Throughout the excitable area movements of the eyelids

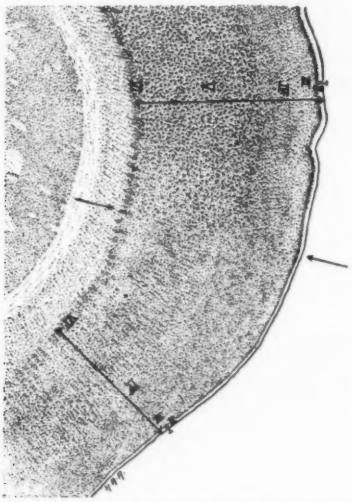

**Figure 3.** Transition from modification 3 of the excitable cortex of *Ornithorhynchus* to the more caudally-lying koniocortex (below). Section cut at 40$\mu$ and stained with cresyl-violet, × 10.

representation is very diffuse: in general it may be said that most anteriorly there were obtained. Martin succeeded in isolating regions which gave maximum closing (his area 2) or opening (his area 3) of the lids, but this refinement was not achieved in these experiments. Extending antero-posteriorly through the middle third of the excitable area is a band which gave movements of the head and forelimb. The was retraction of the head, behind this turning of the head towards the opposite side, and more caudally still advancement of the opposite forelimb. Between the head and forelimb area excitation produced turning of the head combined with advancement of the forelimb. In the right hemisphere of one animal stimulation behind the forelimb area elicited flexion of the opposite side of the body: this result was repeated with certainty only in this particular hemisphere. In the same hemisphere stimulation a little more ventrally produced flexion of the opposite hip associated with erection of the tail: this result could not be repeated with certainty.

In agreement with Martin, the majority of the movements were observed to result from contraction of the panniculus carnosus (*mm. subcutanei colli et trunci*). Careful search at the dorso-medial border failed to reveal Martin's area 4 for retraction of the head, nor was it possible to obtain anything of the nature of organized "digging movements" from the forelimb area such as he described.

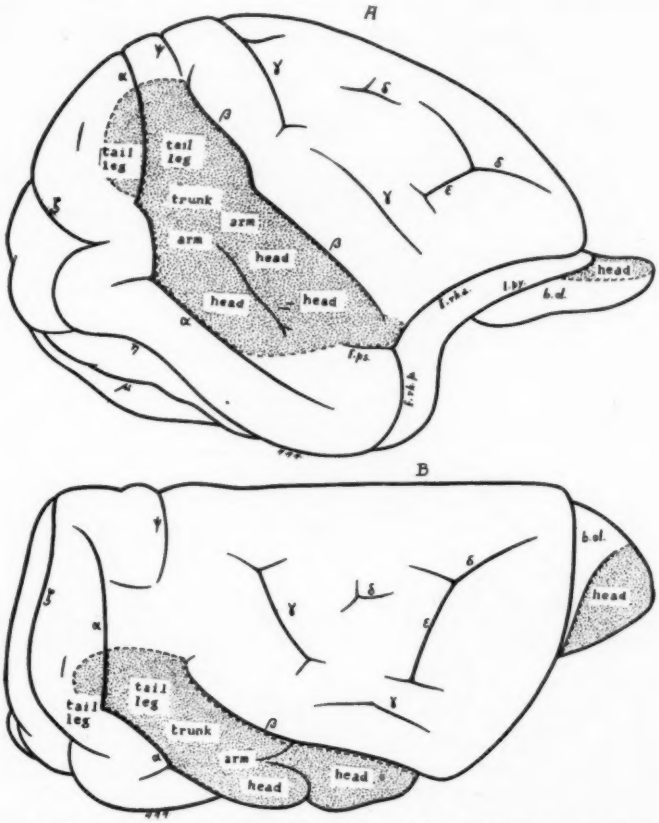


**Figure 4.** The excitable cortex in *Tachyglossus*, × 2½. Note the order of representation and the restriction to the gyrus between sulci α and β. A from the lateral aspect, B from the dorsal aspect.

As may be surmised, such a diffuse representation is not confined to a single cortical formation. Actually no less than three major cortical modifications, which for convenience may be referred to as modifications 1, 2 and 3, are involved (Fig. 2). These pass imperceptibly from 1, through 2, to 3. Modifications 1 and 2 are five-layered and agranular, but 3 presents a typical six-layered granular formation

which ends abruptly, to be replaced by an intensely *granulous* structure of t[illegible] type known as koniocortex (dust cortex) (Fig. 3).

*Tachyglossus.* In this animal the excitable cortex is practically confined [illegible] the gyrus between sulci $\alpha$ and $\beta$, extending from the anterior rhinal fissure belo[illegible]

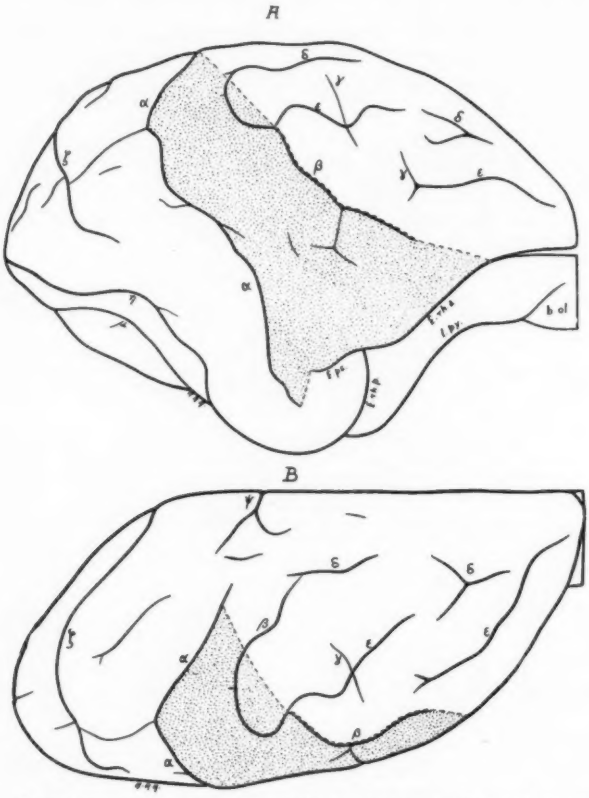


**Figure 5.** Graphic reconstruction of the right hemisphere in *Tachyglossus* to illustrate the distribution of the cortical formation concerned with motor function, × 2¾. A from the lateral aspect, B from the dorsal aspect.

to within two-thirds of the way to the dorso-medial border of the hemisphere (Fig. 4). The arrangement is very characteristic: the cranial end of the body is represented ventrally, the caudal end dorsally. This, however, is a very generalized grouping, the areas are diffuse and merge imperceptibly with their neighbours. The representation is almost exclusively contralateral.

Movements of the tail were chiefly simple erection in the mid-line. In the hindlimb there was usually only flexion of the hip, but occasionally isolated flexion

of the large second digit was observed. In one animal (thyratron tube series) stimulation in this region produced a complete stepping movement of both hindlimbs: strong advancement of the contralateral limb followed by retraction of the ipsilateral; continued stimulation at the same spot reversed the movement, producing weak advancement of the ipsilateral limb followed by retraction of the contralateral. These movements could be repeated indefinitely. Section of the spinal cord through the twelfth thoracic segment abolished all cortical movement of the hindlimbs and tail.

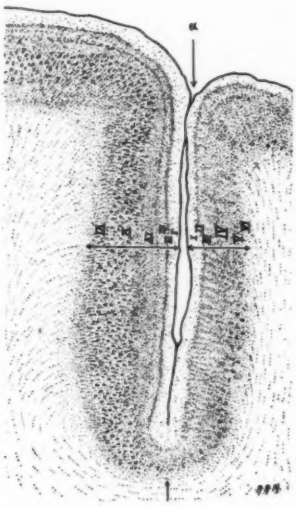

**Figure 6.** Section of a block cut at right angles to sulcus *a* to illustrate the transition from the excitable cortex (to the left) to the more caudally-lying koniocortex. Note that the point of transition occurs abruptly at the floor of sulcus *a*. Section cut at 40$\mu$ and stained with cresyl-violet, × 10.

The trunk movement was generalized flexion of the opposite side of the body. In the forelimb the main response was flexion of the shoulder, but there were occasional isolated movements of individual digits. In the head area stimulation at the upper part evoked retraction of the head; excitation at the lower posterior part elicited turning of the head towards the opposite side, and in the lower anterior part vague head movements difficult to analyse. Between the head and forelimb areas stimulation produced turning of the head towards the opposite side combined with advancement of the opposite forelimb.

Behind sulcus *a* a region was found at the level of the tail and hindlimb area where stronger stimulation produced similar movements of the tail and leg. The remainder of the hemisphere was explored but nowhere else, even on strong stimulation, could any response be elicited. It is possible that the excitable area extended

ventrally beyond the lowest limit indicated in Fig. 4, but this region was inaccessible to the electrode.

Stimulation of the dorsal surface of the olfactory bulb evoked varying responses: in most cases the head was turned towards the side of irritation and there was some snuffling at the nostrils; sometimes, with the same snuffling, the head was turned towards the opposite side; and occasionally there was some movement of the tongue.

In *Tachyglossus* as in *Ornithorhynchus* the majority of the movements depend upon contraction of the panniculus carnosus. This, however, cannot be held responsible for the movements of isolated digits.

The animal in which the excitable cortex was destroyed lived for seventeen days without apparent motor disability. When it was killed the brain was removed and treated according to the usual Marchi technique, but the sections failed to reveal any evidence of descending degeneration.

The distribution of the excitable cortex coincides almost precisely with that of a single cortical area predetermined histologically (Fig. 5) and confirmed from the blocks excised at right angles to sulcus *a*. This cortex, being six-layered and granular, is practically identical in structure with the third modification of the excitable cortex in *Ornithorhynchus;* similarly, it terminates abruptly to be replaced by a granulous koniocortex (Fig. 6). In the case of *Tachyglossus*, however, the point of transition coincides with the floor of sulcus *a* which thus constitutes an accurate line of demarcation. It will be observed that the cerebral cortex in *Ornithorhynchus* as a whole is almost twice as thick as that in *Tachyglossus* (cf. Figs. 3 and 6).

## DISCUSSION.

In considering the results of these experiments the response to stimulation of the olfactory bulb in *Tachyglossus* may be neglected. Such movements are commonly obtained in primitive mammals, and probably represent a reaction to olfactory irritation. They have nothing to do with the excitable cortex as such and are included here only for the sake of completeness. No explanation can be advanced for the response obtained in *Tachyglossus* from stimulation behind sulcus *a* other than by postulating a spread of current. As stated above, the cortical formation behind this sulcus differs entirely from that in front.

Taking the results as a whole it is observed that in *Ornithorhynchus* cortical representation is probably incomplete, is poorly localized, and is spread over a wide area which includes at least three cortical variations. In *Tachyglossus*, on the other hand, localization is much more precise and complete, and is restricted to a single cortical formation which presents a structure similar to that of the third modification in *Ornithorhynchus*. In this respect *Tachyglossus* exhibits the higher degree of organization. In *Ornithorhynchus* representation of parts of the body is disposed more or less antero-posteriorly, whereas in *Tachyglossus*

the disposition is almost vertical. It is rare in primitive mammals to find cortical representaton of the body behind the forelimbs, but in *Tachyglossus* virtually the whole body is represented, and in *Ornithorhynchus* it may be to a lesser extent. Of interest, too, is the situation of the excitable cortex. In *Ornithorhynchus* it extends from the frontal pole almost to the occipital pole, but in *Tachyglossus* it is restricted to the middle third of the dorso-lateral surface. In the majority of primitive mammals the excitable cortex lies close behind the frontal pole, and its caudal situation in *Tachyglossus* is probably unique; indeed, the expanse of cortex lying anterior to the excitable area in this animal is relatively greater than that described for any other mammal, even including man.

In both animals the majority of movements are clearly effected through the panniculus carnosus (*mm. subcutanei colli et trunci*). As is well known, the distribution of this musculature is peculiarly complete in the Monotremata (see Westling, 1889; Ruge, 1895; Huber, 1930, and others), and is well able to produce movements of the head, forelimbs, trunk, hindlimbs and tail. In *Ornithorhynchus* this appears to be the chief muscular mechanism to obtain cortical recognition, but it is difficult to conceive how in *Tachyglossus* it could produce the movements of isolated digits which were observed, and it is necessary to conclude that in this animal the general body musculature is also accorded some cortical representation. The employment of this mechanism in the monotremes is an excellent example of the principle that the cerebral cortex is concerned only with movements, not the means whereby they are effected.

The *m. subcutaneous colli* is innervated through the facial nerve (Huber), while the *m. subcutaneous trunci* is generally believed to be supplied through the anterior thoracic nerves (Westling, Ruge). Since, however, section of the cord through the twelfth thoracic segment abolished movements of the hindlimbs and tail there must be some motor outflow to these through the lower spinal segments. Consideration of the descending pathway involved raises a difficulty. It has been shown (1934) that *Tachyglossus* possesses small pyramidal tracts which probably do not extend below the upper cervical region of the spinal cord; Addens and Kurotsu (1936) would restrict them to the medulla oblongata. These authors revive the suggestion of Fuse (1926) that the Zonalbündel of Koelliker (1901) is pyramidal in nature. However, the only degeneration experiment which was performed to settle this proved unsuccessful.

The structure of the excitable cortex is worthy of comment. In *Tachyglossus*, and in part in *Ornithorhynchus*, it presents a six-layered structure with a well-defined granular fourth lamina (internal granular lamina) whereas motor function is usually associated with the five-layered agranular type of cortex, with or without giant Betz cells. It is of interest to note that in *Didelphys* the excitable cortex is six-layered and granular (Gray, 1924), and that both Brodmann (1909) and Bolton (1914) observed that in the human foetus a six-layered structure precedes the five-layered agranular formation characteristic of the excitable cortex of the adult human. Apparently the motor cortex is primarily six-layered and

granular, and the granular fourth lamina is lost in higher mammals; in human ontogeny this sequence appears to be repeated. In both monotremes the abrupt termination of the excitable cortex caudally, and its replacement by an entirely different formation of only about two-thirds the depth (transition from type 2 to type 5 of von Economo, 1929), is very striking, and recalls to some extent the similar transition in the Primates. There is not, however, sufficient evidence available at present to warrant the establishment of any homologies in this region.

## SUMMARY.

1. The excitable cortex has been determined in four hemispheres in *Ornithorhynchus* and in ten hemispheres in *Tachyglossus*. In the former animal movements were obtained of the eyelids, head and forelimb, and doubtfully of the trunk, hindlimb and tail; in the latter practically the whole body—head, forelimb, trunk, hindlimb and tail—receives some cortical recognition. In both animals the representation is predominantly contralateral.

2. In *Ornithorhynchus* the excitable cortex occupies practically the whole of the dorso-lateral surface of the hemisphere and involves at least three cortical formations; in *Tachyglossus* it is restricted to the lower two-thirds of the gyrus between sulci $\alpha$ and $\beta$, and includes only one cortical formation. The third cortical modification in *Ornithorhynchus* and the whole of the excitable cortex in *Tachyglossus* are alike in being six-layered and granular. Caudally both terminate abruptly, being replaced by an intensely granulous type of cortex.

3. Most of the movements elicited in both animals were produced by contraction of the panniculus carnosus (*mm. subcutanei colli et trunci*) but in *Tachyglossus* movements of isolated digits are probably effected through the general body musculature. Movements of the hindlimbs and tail in this animal are effected through nerves from the lower spinal segments, but the descending pathway involved could not be determined.

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# THE COMBINATION OF DENATURED GLOBIN WITH HAEMATIN, AND WITH PROTO-PORPHYRIN

by

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(From the Walter and Eliza Hall Institute, Melbourne).

*(Submitted for publication 18th March, 1938.)*

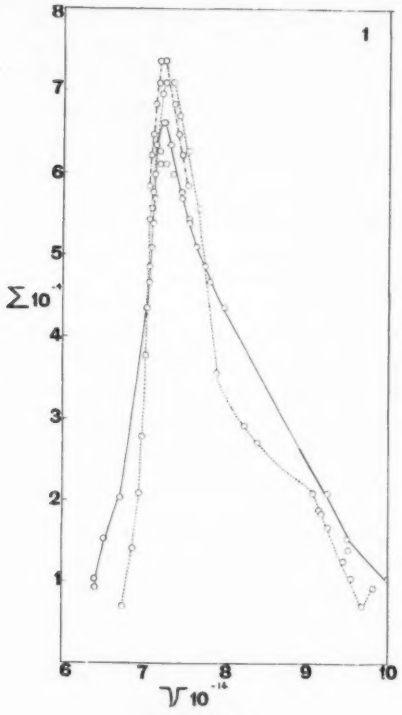
During the work described in a previous paper (Holden, 1937) on the combination of haematin and porphyrins with proteins the writer was unsuccessful in attempts to prepare irrenaturable globin free from all traces of native (or renaturable) globin. Precipitates of irrenaturable globin from faintly alkaline solutions invariably carried down some renaturable globin which, upon appropriate treatment gave rise to methaemoglobin or carbon monoxide-haemoglobin. A spectro-photometric comparison of the amounts of these two substances obtained did not indicate the presence of any protein renaturable only by the use of a reducing agent.

Dr. Lemberg kindly suggested that a procedure, somewhat on the lines of Anson and Mirsky's (1929) original technique for the renaturation of denatured oxyhaemoglobin might be more satisfactory. The solution of denatured globin was therefore kept at pH 6 for several hours before precipitation at its isoelectric point. It was found that two such precipitations coupled with thorough washing of the precipitates yielded a preparation either of denatured globin or of denatured globin parahaematin from which no native protein could be subsequently obtained, either with or without the use of a reducing agent.

Determinations were made of the effect of this denatured globin on the ultra-violet absorption spectra of haematin, proto-porphyrin and haemato-porphyrin. In addition the ultra-violet absorption spectrum of a specimen of denatured globin parahaematin prepared directly from oxyhaemoglobin was determined. The spectrum was practically identical with that of denatured globin parahaematin prepared from haemin and denatured globin.

The experimental details are those of the paper to which reference has been made (Holden, 1937). The denatured globin was that of the rabbit. No appreciable effect was observed in the case of haemato-porphyrin. The spectrum of denatured globin parahaematin was the same as that of alkaline methaemoglobin as recorded by Hicks and Holden (1929) and differed from that of methaemoglobin (see Table 1, Fig. 1). Denatured globin apparently combines with haematin over

a wider range of hydrogen-ion concentrations than is generally believed since th[illegible] spectrum was readily obtained over the range pH 5–9, and with higher concentr[illegible]tions of denatured globin the range could be extended somewhat in either directio[illegible]




**Graph No. 1.**

HAEMATIN.

- - - - - - - Alkaline Methaemoglobin.
——————— Denatured Globin Parahaematin.
— — — — — Denatured Globin Parahaematin (Second specimen).

In the case of denatured globin haemochromogen Holden and Freeman (1929), using more concentrated solutions, found that each equivalent of denatured globin could combine with about nine equivalents of reduced haematin. The instrument used in these researches on parahaematin was adapted for solutions of the order of 0·04 times the concentration of those used by Holden and Freeman (1929). Under these conditions no definite evidence could be found that an equivalent of denatured globin combines with more than one equivalent of haematin. It should, however, be noticed that the absorption coefficient of the maximum in those cases

where an excess of haematin was added was always distinctly higher than the value calculated for a mixture of parahaematin and alkaline haematin.

TABLE 1.

*Haematin and Denatured Globin.*

The molecular absorption coefficients ($\epsilon$) are calculated for the haematin. Those near the iso-electric point are omitted as it is considered that they are fictitious owing to general scattering of light due to a certain degree of aggregation of the particles.

| pH. | Equivalents Haematin per equivalent of denatured globin. | $\nu 10^{-12}$ (maximum). | $\epsilon 10^{-3}$ |
|---|---|---|---|
| 3·5 | 0·7 | 797 | 50 |
| 4·2 | 0·7 | 723 | 62 |
| 4·3 | 0·7 | 721 | 67 |
| 5·9 | 1·6 | 722 | — |
| 6·3 | 1·8 | 721 | — |
| 7·4 | 1·8 | 722 | — |
| 7·7 | 1·9 | 723 | 67 |
| 8·4 | 2·1 | 723 | 64 |
| 8·6 | 1·8 | 723 | 64 |
| 8·9 | 1·2 | 722 | 73 |
| 9·6 | ·9 | 723 | 73 |
| | *Alkaline Methaemoglobin.* | | |
| — | 1 | 730 | 71 |

Another experiment which suggests that one equivalent of denatured globin can combine with more than one equivalent of haematin is as follows:

Two solutions were made up. The first contained 0·9 equivalents of haematin per equivalent of denatured globin at pH 9·5. The second contained twice as much haematin (1·8 equivalents) as the first per equivalent of denatured globin at pH 9·3. The solutions were in all other respects precisely similar. The second solution, although nearer to the iso-electric point of denatured globin, was very much less opalescent than the first. The most reasonable hypothesis is that some of the additional haematin had combined with the protein, displacing the iso-electric point of the latter towards the acid side. Parahaematin from oxyhaemoglobin by denaturation has an iso-electric point (pH 6·8) much more acid than that of the corresponding denatured globin (pH 8·1).

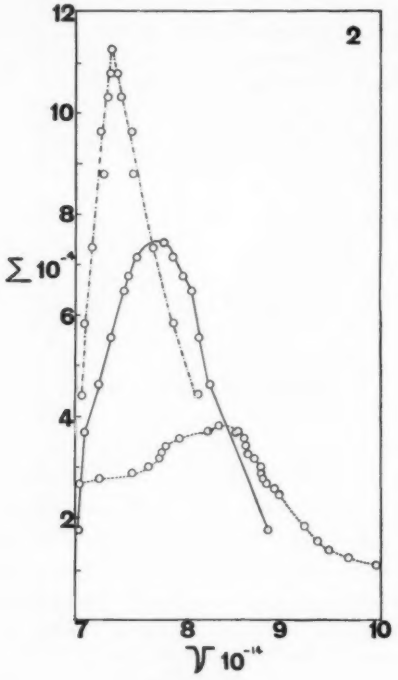
TABLE 2.

*Proto-porphyrin and Denatured Globin.*

| pH. | Equivalents of porphyrin per equivalent of denatured globin. | $\nu 10^{-12}$ (maximum). | $\epsilon 10^{-3}$ |
|---|---|---|---|
| 4·5 | 1·4 | 803 | 42 |
| 5·4 | 1·8 | 799 | 50 |
| 6·4 | 1·8 | 775 | — |
| 9·0 | 1·8 | 771 | 59 |

Only the last two show much evidence of combination.

The spectrum of proto-porphyrin-denatured globin does not resemble that of any other compound of this porphyrin observed in these studies. The compound appeared to be formed best in faintly alkaline solution. In acid solutions only a small proportion of the porphyrin combined with the protein (see Table 2, Fig. 2).




**Graph No. 2.**

PROTOPORPHYRIN.

- - - - - - - Protoporphyrin.
— — — — — Protoporphyrin and Native Globin.
——— Protoporphyrin and Denatured Rabbin Globin.

In other experiments both haematin and proto-porphyrin were added successively to a limited amount of denatured globin. It was found that even when added first the porphyrin was eventually displaced by the haematin.

## SUMMARY.

The ultra-violet spectrum of denatured globin parahaematin resembles that of alkaline methaemoglobin. That of proto-porphyrin-denatured globin does not resemble that of other known compounds of this porphyrin.

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# THE SUBSTITUTION OF METHIONINE FOR CYSTINE IN THE DIET OF GROWING RATS

by

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During the last few years it has been shown repeatedly that the cystine in the diet may be replaced in part by methionine.

The growth of young rats, arrested by feeding on a diet deficient in sulphur-containing amino acids, and particularly in cystine, was resumed when either d- or l-methionine or l-cystine was fed (Jackson and Block, 1932, 1938; Weichselbaum, 1935). When the dietary deficiency was aggravated by feeding bromobenzene, cholic acid or napthalene, either methionine or cystine was effective in restoring growth (White and Jackson, 1933, 1935; White, 1935–6; Stekol, 1937b). However, none of these experiments proved that cystine may be entirely eliminated from the diet. The experimental dietaries used were deficient, but not completely lacking in this amino acid: in particular, the B vitamin supplements contributed an unknown and possibly significant amount to the diet.

The capacity of methionine to carry out some of the functions of cystine has been shown by experiments of a different type. Methionine, as well as cystine, was observed to bring about an increased synthesis of taurocholic acid when fed with cholic acid to dogs on a fasting régime (Virtue and Doster-Virtue, 1937). It has been found that bromobenzene, fed to animals, leads to an increased excretion of urinary sulphur, partly as ethereal sulphate and partly as an organic sulphur compound, p-bromo-phenyl-mercapturic acid. The partition of sulphur in the urine undoubtedly depends on the diet, an abundant supply of cystine favouring excretion of the mercapturic acid. Methionine was observed to have the same effect as cystine in this regard (White and Lewis, 1932; Stekol, 1937a, 1938). In cystinuria the excretion of cystine in the urine was found to be increased by feeding either cystine or methionine, though cystine itself was oxidized under such conditions (Brand and Cahill, 1934; Brand, Cahill and Harris, 1933–4, 1935; Lewis, 1935; Lewis, Brown and White, 1936; Brand, Block, Kassell and Cahill, 1937).

Considerable interest was aroused by the suggestion of Marston and Robertson (1928) that, as merino wool keratin contains about 13 p.c. of cystine and, so far as is known, plant proteins have comparatively little cystine in their make-up, the growth of wool fleece by high producing merino sheep might, when the protein intake was small, be limited by the ingestion of cystine in the fodder. If methionine can be converted into cystine in the living organism the potential source in the diet of the cystine for wool growth is greatly increased, being the sum of the cystine and methionine in the fodder.

Marston (1935) investigated this problem. He found that when wool production by the merino ewe was limited by a low protein intake l-cystine added to the diet brought about a material increase in wool growth. In order to circumvent destruction of cystine by the rumen flora he injected the equivalent of cysteine subcutaneously and observed a much greater increase. Injection of the equivalent of methionine under identical circumstances brought about an increase so small as to lead him to consider it to be of doubtful significance. He pointed out the possibility that on injection into the mildly oxidizing environment of the subcutaneous tissues the cysteine became precipitated as sparingly soluble cystine, and in this form was absorbed slowly enough to escape destructive deamination, and was thus efficiently utilized by the follicles. The rapid absorption of the soluble methionine injected under like circumstances may well have accounted for the difference noted.

The experiments recorded here were undertaken in an attempt to determine unequivocally whether cystine is an essential dietary constituent or whether it may be derived from methionine *in vivo*. Since they were begun Womack, Kemmerer and Rose (1937) have published experimental findings which establish the fact that cystine is not one of the essential amino acids: methionine on the other hand has been shown to be essential. (See also White and Beach, 1937.) While confirming the work of Rose and his collaborators the following experiments also show that methionine may be converted into the cystine necessary for hair growth in the rat, and imply that this transformation may also be accomplished by the sheep.

## EXPERIMENTAL.

Young male rats weighing between 40 and 50 grams were confined in separate cages and fed upon a basal diet containing only a trace of cystine. The sole source of protein was a hydrolysate of casein from which the cystine had been precipitated as the cuprous mercaptide. The diet, which was fed ad lib. had the following composition:

| | |
|---|---|
| Dextrinized starch | 75 gms. |
| Cottonseed oil | 15 „ |
| Lard | 3·75 „ |
| Salt mixture [Jansen (1931)] | 6·25 „ |
| Casein hydrolysate | 10 „ |
| l-tryptophane | ·50 „ |
| With or without l-histidine HCl | ·40 „ |

The casein hydrolysate was prepared as follows:

Fifty litres of fresh separated cow's milk were diluted to 250 litres with water in a glass-lined vat, and the reaction cautiously adjusted to pH 4·5 by slowly injecting a stream of decinormal acetic acid under the surface of the solution while vigorously agitating it with an efficient mechanical stirrer in the bottom of the vat. Stirring was discontinued an hour after the isoelectric point had been reached and the very finely divided casein allowed to settle over night. The supernatant liquid was removed as completely as possible next morning and the precipitate washed twice by stirring for an hour each time with 250 litres of water to which sufficient acetic acid was added to bring the whole suspension to pH 4·5, allowing to settle and decanting the washing water. The precipitate was then again suspended in 250 litres of water and dissolved by the addition of NaOH until the reaction was about 6·0. The casein was then precipitated and washed as before. After five such precipitations and washings the protein was found to be free from any trace of reducing sugars. The large bulk of precipitate was then separated from the major part of the adherent water by means of a filter press and the cakes transferred to another vat, where they were broken up and washed with several changes of absolute alcohol until the last traces of fat were removed. It was freed from the bulk of the alcohol by returning to the original vat and washing twice in the manner outlined above. It was then redissolved, precipitated and washed. The wet casein was then suspended in a small bulk of water, stirred, and sufficient concentrated sulphuric acid added and the volume adjusted until the suspension contained 10 per cent of casein in 6 N. acid. Five hundred grams of powdered tin were added and the casein hydrolysed by boiling under a reflux for 24 hours. The sulphuric acid content was then reduced to approximately 1·5 N. by the addition of boiling saturated Baryta. The precipitate of $BaSo_4$ and tin which remained undissolved was filtered off on large Buchners and well washed with boiling water, the washings being added to the filtrate. A further 50 gm. of tin were added to the filtrate to ensure reducing conditions. About 13 litres of almost colourless solution, containing 18·9 mg. of nitrogen per ml. resulted.

The cystine was then removed as the copper mercaptide (Vickery and White, 1932–3). Precipitated cuprous oxide was added to the solution while stirring vigorously with an electric stirrer, until a definite excess was evidenced by a persistent reddish colour in the suspension. The stirring was continued at room temperature for some hours and the whole cooled to 10°C. over night. Next day the acidity was reduced to pH 4·5 by a further addition of saturated Baryta, the reaction being checked by glass electrode measurements, and the mass surrounded the while with ice so as to ensure that the temperature did not rise appreciably above 30°C. The result was a light chocolate coloured sludge suspended in a deep blue solution. This was stood for a further 24 hours in the freezing chamber. While still cold it was filtrated on large Buchners and the mass of $BaSO_4$, which contained the cuprous mercaptide and the excess copper oxide, was suspended in water, reduced to pH 4·5 with sulphuric acid, cooled to 10°C. overnight, and refiltered and washed, the washings, etc., being added to the original filtrate. The copper was then removed by shaking under an atmosphere of hydrogen sulphide and filtering off the copper sulphides. The excess $H_2S$ was removed by boiling *in vacuo*.

The remaining sulphuric acid was then quantitatively removed with barium hydroxide and the filtrate taken to dryness in a Newmann vacuum still.

The $BaSO_4$ cuprous oxide precipitate was re-extracted with cold water at pH 4·4 and re-precipitated with $Cu_2O$ and Sn at 5°C. The Cu was removed from the solution by $H_2S$ and the $SO_4$ precipitated by treating with $BaOH_2$. The resulting filtrate was taken to dryness, analysed for -SH and -S-S- and added to the main bulk.

Precautions were taken to prevent the destruction of tryptophane. Nevertheless it was added to the diet to ensure its presence in adequate amount. When the experiment was under way histidine also was added to the diet of certain of the animals. There seemed to be no difference in the growth rate of the rats receiving added histidine and those without it.

For the first four weeks the uncooked diet was fed. Thereafter some of the rats continued to receive the raw diet while for others it was cooked by heating in a small steam-jacketed oven.

Throughout the experiment care was taken that the rats receiving methionine were treated in exactly the same way as the corresponding ones receiving cystine.

The fat-soluble vitamins were supplid as four drops daily of codliver oil, the water-soluble B vitamins as extracts of wheat germ and liver.

## PREPARATION OF VITAMIN B EXTRACTS.

*Wheat germ extract:*

Wheat germ was extracted three times with 90 p.c. alcohol at room temperature, using 2 litres of alcohol per kg. of wheat germ for the first extraction and 1 litre for the second and third extractions. The combined filtered extracts were concentrated under reduced pressure to drive off the alcohol, and diluted so that 1 ml. was equivalent to 1·5 gm. of wheat germ. The daily dose was 1 ml.

*Liver extract:*

2,000 grams of minced ox liver were twice extracted by boiling with an equal weight of water and filtering. The filtrate was acidified with HCl, and stirred for 1 hour with 100 gm. of B.D.H. fuller's earth. This was allowed to settle and the supernatant liquid decanted and stirred again with 100 gm. of fuller's earth. The earth was freed from the supernatant solution by centrifuging, and washed with distilled water till it began to go into colloidal solution. The vitamins were eluted by stirring the earth for 1½ hours with 4 litres of dilute pyridine and methanol, and centrifuging. The earth was then washed with dilute methanol. The eluting and washing solutions were concentrated under reduced pressure and an equal volume of methanol was added to precipitate the fuller's earth which had gone into colloidal solution. This was filtered off, washed with methanol and the filtrate and washings concentrated to a small volume under reduced pressure. In this process the methanol and the bulk of the pyridine were driven off (György, Kuhn and Wagner-Jauregg, 1934; Birch and György, 1936). The concentrated solution was diluted to 800 ml. The daily dose was 2 ml ≡ 5 gm. of liver.

The growth-promoting power of the vitamin supplements was tested with rats whose diet differed from the above basal diet only in containing 20 p.c. of purified casein and ·2 p.c. of l-cystine. The vitamin supply was found to be entirely satisfactory for the purposes of this experiment, permitting growth of upwards of 2 grams a day and maintaining the rats in good health. Growth in the other experimental groups was at about half this rate, so that it was obviously not the vitamin supply which limited it.

To test the capacity of methionine to replace cystine three litters, each of five male rats, were used, and were divided into five groups of three rats. A rat from each litter was allotted to every group by the use of Tippett's random numbers (1927). The rats in Group A were maintained on the basal diet, those in Groups B and D received a cystine supplement after the first week, and, and those in Groups C and E a supplement of methionine.

The cystine was pure l-cystine, of which 20 mg. daily were fed to each rat in its vitamin supplement till the animal reached a weight of 80 grams. The amount was then increased to 30 mg. The methionine was pure dl-methionine (Fraenkel and Landau) and the amount fed daily was 25 mg. increased to 37·5 mg. when the rat reached 80 gm. weight. The rats were weighed three times a week.

After 9 or 11 weeks on the experimental diets they were killed with gas and the fleeces removed by plucking after plunging the carcasses in hot water for 2 or 3 minutes (Smuts, Mitchell and Hamilton, 1932). The fleeces were then carefully and repeatedly washed with boiling water, boiling alcohol and cold ether, in that order, and dried to constant weight in a vacuum oven.

They were subsequently hydrolysed by boiling for three to four hours under reflux with approximately 5·5 N HCl, and the cystine content was determined by the method of Folin as modified by Lugg (1932). The cystine content of the fleece of those rats which had received methionine was determined also by Lugg's modification of Sullivan's method (Lugg, 1933). This was done to make certain that the substance estimated as cystine was cystine itself and not homo-cystine. There was little difference between the results obtained by the two methods.

Six young male rats, selected at the same time as those for the growth experiment, were killed and the weight and cystine content of the fleeces were determined. The animals were approximately the same age and weight as the experimental rats but were from different litters.

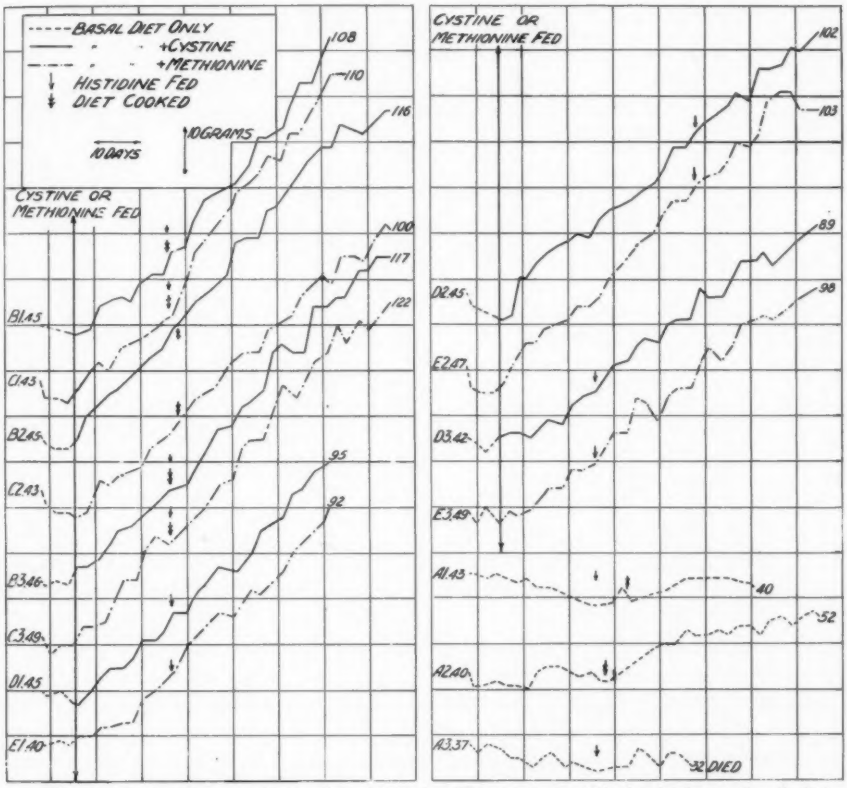


**Fig. 1.** *Growth Curves of Experimental Rats.* The numbers at the beginning and end of the curves are the initial and final weights of the rats in grams.

The experimental results are given in Fig. 1 and in Tables 1 and 2. It is evident that the rats receiving methionine grew at the same rate as those receiving cystine, and that the cystine content of their fur was as high, 14·2 p.c.

The fur of rats on a casein diet unsupplemented with cystine was found to contain between 11·1 and 13·5 p.c. of cystine, with a mean value of 12·8 p.c., while the average cystine content of the fur of rats whose diet contained added cystine was 14·1 p.c. ( Dawbarn,1936). Lightbody and Lewis (1929) and Smuts, Mitchell and Hamilton (1932) also found that the rat's fur reflects the cystine content of its diet.

The high cystine content of the fur of all the rats fed either cystine or methionine is evidence for the adequacy of the supply of cystine or some substance capable of conversion to cystine in the body.

TABLE 1.

| Rat. | Litter No. | Supplement. | Days on experimental diet. | Initial weight. (gm.). | Final weight. (gm.). | Increase in weight. (gm.). | Increase in weight per week. (gm.). |
|---|---|---|---|---|---|---|---|
| A1 | 1 | None | 63 | 43 | 40 | −3 | |
| A2 | 2 | „ | 77 | 40 | 52 | 12 | 1·1 |
| A3 | 3 | „ | 77 | 37 | 32 (died) | | |
| B1 | 1 | l-cystine | 64 | 45 | 108 | 63 | 7·0 |
| B2 | 2 | „ | 77 | 45 | 116 | 71 | 6·5 |
| B3 | 3 | „ | 77 | 46 | 117 | 71 | 6·5 |
| D1 | 1 | „ | 63 | 45 | 95 | 50 | 5·6 |
| D2 | 2 | „ | 77 | 45 | 102 | 57 | 5·2 |
| D3 | 3 | „ | 77 | 42 | 89 | 47 | 4·3 |
| **Mean** | | | | | | | 5·81 |
| C1 | 1 | dl-Methionine | 64 | 43 | 110 | 67 | 7·4 |
| C2 | 2 | „ | 77 | 43 | 100 | 57 | 5·2 |
| C3 | 3 | „ | 77 | 49 | 122 | 73 | 6·6 |
| E1 | 1 | „ | 63 | 40 | 92 | 52 | 5·8 |
| E2 | 2 | „ | 77 | 47 | 103 | 56 | 5·1 |
| E3 | 3 | „ | 77 | 49 | 98 | 49 | 4·5 |
| **Mean** | | | | | | | 5·76 |

While receiving the experimental diets four of the rats (three fed methionine and one cystine) shed a part of their fur—that on the belly and the lower part of the back near the tail. No change could be observed in the rest of the fleece, and the whole skin appeared perfectly healthy. One rat, C2, grew a new short coat of fur only to lose it again a few days later. At the time of killing the affected areas were not completely denuded but carried fur about a millimetre in length. The weight of the fleece was naturally much less than that of the other rats.

The loss of fur was probably due to the low level of protein in the diet. Hartwell (1924, 1925) reported a similar shedding of part of the fleece in rats, which she attributed to protein deficiency. The high percentage of cystine in the fleece makes it unlikely that there was any shortage of this substance. Cystine does not increase hair growth when it is a deficiency of some other amino acid which is limiting the growth-promoting capacity of the diet (Smuts, Mitchell and Hamilton, 1932).

An attempt has been made to calculate the amount of cystine ingested by the rats on the basal diet supplemented with methionine, and to compare this with the cystine stored in the carcase and fleece.

TABLE 2.

| Rat. | Supplement. | Weight of Rat. (gm.). | Weight of fleece. (gm.). | Cystine in fleece in mg. (Folin-Lugg). | Cystine in fleece in mg. (Sullivan-Lugg). | P.c. cystine in fleece. (Folin-Lugg). | Mg. fur per sq. cm. surface†. | Mg. fur per gm. body weight. |
|---|---|---|---|---|---|---|---|---|
| G1 | | 42 | ·5015 | 67·4 | | 13·4 | 4·23 | 11·9 |
| G2 | | 49 | ·5323 | 70·4 | | 13·2 | 4·08 | 10·9 |
| G3 | | 42 | ·4884 | 66·4 | | 13·6 | 4·11 | 11·6 |
| G4 | | 47 | ·5473 | 67·5 | | 12·3 | 4·30 | 11·6 |
| G5 | | 42 | ·5385 | 64·9 | | 12·0 | 4·53 | 12·8 |
| G6 | | 43 | ·5200 | 63·4 | | 12·2 | 4·31 | 12·1 |
| A1 | | 40 | ·4161 | 48·0 | | 11·5 | 3·61 | 10·4 |
| A2 | | 52 | ·6540 | 81·5 | | 12·5 | 4·84 | 12·6 |
| A3 | | 32 | ·3560 | 42·9 | | 12·05 | 3·53 | 11·1 |
| **Mean** | | | | | | 12·0 | | |
| B1 | l-cystine | 108 | 1·4007 | 191 | | 13·6 | 6·69 | 13·0 |
| B2 | „ | 116 | 1·7425 | 248 | | 14·2 | 7·97 | 15·0 |
| B3 | „ | 117 | 1·7233 | 254 | | 14·7 | 7·84 | 14·7 |
| D1 | „ | 95 | 1·4756 | 204 | | 13·8 | 7·61 | 15·5 |
| D2 | „ | 102 | ·5929* | 80·6 | | 13·6 | | |
| D3 | „ | 89 | 1·3258 | 193 | | 14·6 | 7·11 | 14·9 |
| **Mean** | | | | | | 14·1 | | |
| C1 | dl-Methionine | 110 | 1·7454 | 252 | 250 | 14·4 | 8·24 | 15·9 |
| C2 | „ | 100 | ·8484* | 119 | 125 | 14·0 | | |
| C3 | „ | 122 | 1·9062 | 270 | 277 | 14·2 | 8·46 | 15·6 |
| E1 | „ | 92 | 1·1320* | 160 | 163 | 14·1 | | |
| E2 | „ | 103 | 1·5488 | 218 | 221 | 14·1 | 7·61 | 15·0 |
| E3 | „ | 98 | ·6209* | 88·7 | 86·1 | 14·3 | | |
| **Mean** | | | | | | 14·2 | | |

* The rats shed the fur on the belly and lower back.
† The surface area was calculated from the body weight by the use of Lee's formula I (1929).

The disulphide compounds present in the casein hydrolysate and in the liver extract after hydrolysis were determined by Lugg's modification of Folin's method, and assumed to be cystine. It was not possible to determine the disulphides in the extract of wheat germ. The amount of humin formed during the hydrolysis required to liberate cystine for estimation was so great as to make any attempt at determining cystine quite valueless (Lugg, 1933). The total sulphur was therefore determined by combustion in the bomb of the Emerson fuel calorimeter, and the assumption made that the whole of the sulphur came from cystine, since it was considered better to over-estimate than to under-estimate the cystine content.

The cornflour from which the dextrin was prepared contained 0·068 p.c. of nitrogen. Conceivably, therefore, it contained cystine. There was too little sulphur present for a satisfactory determination, and an estimation of cystine itself was out of the question. The cystine which it might contain was therefore calculated on the assumption that the nitrogen was present as protein containing 2 p.c. of cystine.

TABLE 3.

| Rat. | Intake of basal diet. (gm.). | Cystine Intake in mg. (estimated). Dextrin‡. | Casein†. | Liver† extract. | Wheat germ§ extract | Total. | Cystine in fleece (less 70 mg.). (mg.). |
|---|---|---|---|---|---|---|---|
| C1 | 412 | 23·9 | 4·0 | 16·65 | 16·0 | 60·6 | 182 |
| *C2 | 504 | 29·2 | 4·9 | 17·75 | 17·0 | 68·9 | 49 |
| C3 | 539 | 31·3 | 5·3 | 17·15 | 16·4 | 70·1 | 200 |
| *E1 | 336 | 19·5 | 3·3 | 15·75 | 15·1 | 53·7 | 90 |
| E2 | 506 | 29·3 | 5·0 | 17·75 | 17·0 | 69·0 | 148 |
| *E3 | 454 | 26·3 | 4·4 | 17·15 | 16·4 | 64·3 | 18·7 |

* The rats shed part of their fur.
† Estimated by Lugg's modification of Folin's method.
‡ Calculated from the nitrogen content, assuming the nitrogen to be present as protein containing 2 p.c. cystine.
§ Assuming the whole of the sulphur to be present as cystine.

The results of these calculations are recorded in Table 3. The figures for total cystine intake must be regarded as maximal. The actual amount ingested may have been considerably less.

The cystine stored in the fleeces of the rats during the experiment was calculated by determining the cystine content at the end, and subtracting from it 70 mg., the largest amount of cystine found in the fur of the young rats.

The cystine in the carcases has not yet been determined, but an approximation to the amount present may be made by calculation.

The cystine content of muscle proteins has been reported as between 0·8 and 1·7 p.c. (Mitchell and Hamilton, 1929). Bailey (1937) found 1·96 p.c. of cystine in rabbit myogen and 0·77 and 0·72 p.c. in rabbit and dog myosin. Marston (1932) by determination of total sulphydryl in reduced enzyme digests of sheep muscle protein estimated that the whole protein contained approximately 1·25 p.c. of cystine. Thus body protein may be expected to contain about 1 p.c. of cystine.

Rats fed on a diet containing 7 to 12 p.c. of casein with or without extra cystine were found to contain between 2·4 and 2·6 p.c. of protein nitrogen, exclusive of that in the fleece (Dawbarn, 1936).

If each 10 gm. weight increase in the present experiments represented a storing of 0·24 gm. of nitrogen, equivalent to 1·50 gm. of protein containing 1 p.c. of cystine, then the rats fed on the basal diet and methionine would increase their store of body cystine exclusive of that in the fleece by from 73 to 110 mg. This is more than the cystine ingested. When no shedding of fur occurred the amount of cystine stored in the fleece alone was two or three times as great as that ingested. Conversion of methionine to cystine must therefore have taken place.

## SUMMARY.

Dl-methionine proved as efficient as l-cystine in promoting the growth of rats fed on a cystine-deficient diet, and for the growth of fleece.

The amount of cystine stored in the fleece of the rats fed on a basal diet supplemented with methionine was very much greater than that ingested by the animals.

It follows that part of the methionine was converted into cystine.

## ACKNOWLEDGMENTS.

My thanks are due to Mr. H. R. Marston who suggested the investigation of this problem and who prepared the cystine-free casein hydrolysate used as the main nitrogen source in these experiments.

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# UROBILINS AND UROBILINOGENS

by

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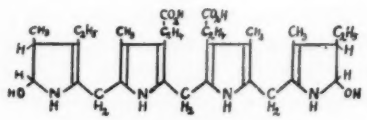
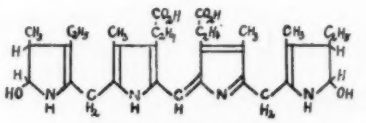
When bilirubin is reduced by sodium amalgam it yields a leuco-compound $C_{33}H_{44}O_6N_4$, which was isolated by H. Fischer in 1911 and called mesobilirubinogen (formula III), the prefix "meso" indicating that the vinyl side chains of bilirubin have become reduced to ethyl. The shorter name mesobilinogen, which

I.



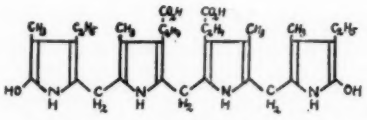
Urobilinogen (stercobilinogen).

II.



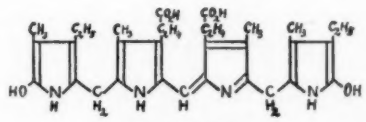
Urobilin (stercobilin).

III.



Mesobilinogen (mesobilirubinogen).

IV.

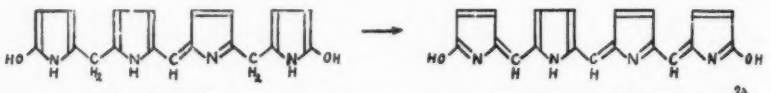


Urobilin-IVα.

is also in use, is preferable, because it is in better harmony with the fact that this substance is the chromogen of other bile pigments of the meso-type as well as of mesobilirubin. The oxidation of mesobilinogen by atmospheric oxygen produces a yellow pigment, urobilin—IXa[1], which is very similar in properties to the urobilin of urine and faeces. It has been isolated in the crystalline state and synthesized by Siedel (1936), and this synthesis has proved it to possess the formula

[1] "IX" designates that the compound has the same arrangement of side chains as mesoporphyrin IX and "IXa" that is is derived from mesoporphyrin IX by removal of the CH—group α, which stands between the two non-carboxylated pyrrole rings.

IV, already suggested by Lemberg (1934) on the basis of the fact that the oxidation of the substance leads to mesobiliverdin (with CH in the centre of the molecule), but does not yield mesobilirubin (with $CH_2$ in the centre of the molecule):




(The side chains of the formulae have been omitted).

Until recently the urobolinogen from urine and faeces was believed to be identical with mesobilinogen, and the natural urobilin with the substance now called urobilin—IXα. This assumption was based on the isolation by H. Fischer and Meyer-Betz of crystalline mesobilinogen from a pathological urine in 1911. Long before this Hopkins and Garrod (1896, 1897) had found some differences between natural urobilin and "hydrobilirubin" (impure urobilin—IXα), but their observations were neglected, after Fischer had shown that the presumptive difference in nitrogen content of the two substances was due to impurities admixed to the natural urobilin of the authors. It will be seen from the present paper that some of the differences noticed by the English authors were genuine.

In 1932 Watson, working in Fischer's laboratory, succeeded in isolating urobilin from faeces in the crystalline state and described experiments which made it unlikely that its reduction yielded mesobilinogen. He called the substance stercobilin (the name given by Van Lair and Masius to faecal urobolin), because he believed it to be different from the urobilin of urine, the close relationship of which with mesobilinogen he took as proved. He corrected this error in 1933 when he isolated the same substance from various pathological urines. The identity of urobilin from faeces and from urine was confirmed by Heilmeyer and Krebs (1934) and by Watson (1935)[3]. Fischer and his school retained the name stercobilin for

[2] A somewhat different formula had been suggested for the biliverdins by Lemberg and Barcroft in 1932 (Proc. Roy. Soc. (London) B, 110, p. 365), but had been later abandoned in favour of the formula of Fischer, given above. It is now again suggested by Fischer and Halbach (1936), but we consider the evidence brought forward by Lemberg in 1934 (Biochem. J., 28, p. 986) in favour of Fischer's formula as stronger than that now brought forward by Fischer in favour of the formula of Lemberg and Barcroft.

[3] The statement of Siedel (Zeitschr. physiol. Chem., 242, p. 114 (1936)): "Während sich nun sowohl Heilmeyer und Krebs, wie C. J. Watson für die Identität des Sterkobilins mit dem Urobilin aussprachen, entdeckten H. Fischer, Halbach und Stern als einen entscheidenden Unterschied die optische Aktivität des Sterkobilins" is misleading. It can only mean that Fischer et al. are said to have corrected an erroneous assumption of the other authors and that in the optical activity they have discovered a decisive difference between stercobilin and urinary urobilin. The latter statement has indeed been quoted in the literature. In fact Heilmeyer and Krebs and Watson have proved the identity of the common type of urinary urobilin with stercobilin, and the discovery of the optical activity of stercobilin (urobilin) by H. Fischer et al. only confirmed the non-identity of it with urobilin-IXα. The polemic note of Siedel against one of us (R.L.) in Zeitschr. physiol. Chem., 245, p. 265 (1937) contains several statements of a similarly misleading nature. It will be shown in a forthcoming paper that Siedel's formula for mesobiliviolin is incorrect.

this substance. In 1934 the question was still far from being cleared. Heilmeyer considered urobilinogen (stercobilinogen) as identical with mesobilinogen, and Watson also in 1933 and still in 1935 believed mesobilinogen to be the mother substance from which stercobilin was derived by oxidation (whether by oxidation *in vivo* or *in vitro* Watson did not say). Lemberg (1934) gave for the first time conclusive evidence that urobilinogen from urine as well as from faeces differed from mesobilinogen as urobilin from urobilin-IX$\alpha$, and that mesobilinogen cannot be the mother substance of urobilin. By ferric chloride urobilinogen was oxidized to urobilin only and not to the blue oxidation products produced by this oxidizing agent from mesobilinogen or urobilin-IX$\alpha$. At that time the correct analytical composition of urobilin was not known, and the explanation in terms of structure given to account for the difference in behaviour of urobilin and urobilin-IX$\alpha$ (two CO groups in urobilin instead of two CH groups in urobilin-IX$\alpha$) was incorrect. The difference itself was confirmed by Fischer and Halbach (1936) and by Siedel (1936). Fischer, Halbach and Stern (1936) discovered as an additional difference the optical activity of urobilin (stercobilin), while all other bile pigments are optically inactive. They also found that urobilin can be oxidized to glaucobilin (mesobiliverdin) by heating in conc. sulphuric acid, and this fact, together with more recent analyses indicating that urobilin has six oxygen atoms like urobilin-IX$\alpha$ and is richer in hydrogen than this substance, has led to the formula II given above for urobilin (stercobilin) and of formula I for urobilinogen (stercobilinogen).

In spite of these publications the majority of biochemists and physiologists have not yet become aware that the time-honoured belief in the identity of urobilinogen and mesobilinogen is erroneous.[4] The reason for this confusion is the use of an unsuitably chosen and misleading nomenclature by Fischer and his school. Based on the occurrence of mesobilinogen in a few pathological urines (Fischer and Meyer-Betz, 1911; Watson, 1936) Fischer maintains the name urobilinogen for mesobilinogen, and Siedel even terms urobilin-IX$\alpha$ "the natural urobilin", while the substances occurring exclusively or predominantly in normal urine and in the great majority of pathological urines are termed stercobilin and stercobilinogen. This practice is apt to hide rather than to reveal the new facts, and we return, therefore, to the original name "urobilin" of Jaffé whose conception of the identity of urobilin and stercobilin can be shown to be essentially correct. Watson (1936) believes that urine contains both urobilin and urobilin-IX$\alpha$, but his view, like the earlier view of Fischer, is supported by the investigation of only one urine (in a case of haemolytic jaundice).

The existence of a "pathological" or "febrile" urobilin was long ago claimed by MacMunn (1881, 1885, 1889) and by Eichholz (1893), but Hopkins (1893) and Garrod and Hopkins (1896) have shown that the differences observed by these

[4] In the report on Animal Pigments in the Annual Review of Biochemistry, 5, p. 479 (1936), for example, it is reported that urobilin and stercobilin are reduced to mesobilinogen.

authors were mainly due to admixture of other pathological pigments (porphyrins, urorosein). From the reading of MacMunn's rather confused description one obtains the impression that he may have occasionally noticed real and characteristic differences, but that he was led astray by a tangle of presumptive differences due to the presence of other pigments and to the accidental absence or presence of metals such as zinc and copper which combine with urobilin.

The non-identity of urobilin and urobilin-IXα and the occasional occurrence of the latter substance in the urine are of great physiological importance, and may be of clinical importance. It is evidently necessary to re-examine a great deal of earlier evidence concerning the physiology of urobilin. Is the urobilin obtained from bilirubin by the action of intestinal bacterial synergism *in vitro* (Kämmerer, 1924) urobilin or urobilin-IXα, and what is the urobilin excreted by the bile fistula dog when fed with bile? If urobilin-IXα is present in the urine of a patient, is it also present in his faeces, or is it of extraintestinal origin? Has the occurrence of urobilin-IXα in the urine a pathological significance? For the study of all these questions it is essential to have a simple micro-method allowing one to distinguish between the two compounds, and methods to measure their relative and absolute quantity. To these problems our paper gives an introductory contribution. We have also investigated a large number of pathological urines with one of the new methods.

## EXPERIMENTAL.

*Preparations.*

*Urobilin-IXα* was prepared in two ways:

(a) According to the method of Siedel (1936) from mesobilinogen. Some admixed mesobiliviolin was removed by chromatographic adsorption to talc from a chloroform solution. The zones were developed by a mixture of two volumes of chloroform and one volume of methyl alcohol. A small yellow zone and a violet zone migrated first through the column, while the dark-yellow upper zone retained urobilin-IXα. This was eluted by methyl alcohol, and its hydrochloride now crystallized from acetone. Its properties agreed with those described by Siedel.

(b) By exposing a petrol-ether solution of mesobilinogen to direct sunlight. The urobilin-IXα formed was taken up by water and extracted from it by chloroform. With regard to the properties described in the present paper no differences were found between preparations a and b.

*Stercobilin* (faecal urobilin) was obtained from normal human faeces, from faeces of a patient with pernicious anaemia and from rats' faeces[5] by extraction with glacial acetic acid-ether mixture and further treatment as described by Watson (1934). Stercobilin hydrochloride was crystallized from acetone. No difference was found between the preparations from the various faeces.

*Urinary urobilin* was extracted from normal urine by chloroform after half-saturation with ammonium sulphate. A larger amount was obtained from urine in a case of Banti's disease, and this could be subjected to a more vigorous purification. It was extracted from the chloroform solution by N/10 sodium hydroxide and brought into ether with acetic acid. Water extracted the larger part of it from the ether solution and ultimately the pigment was extracted from the

[5] Rats' faeces contained a remarkably large amount of porphyrins, which consisted of protoporphyrin (predominant), of coproporphyrin and of deuteroporphyrin.

aqueous solution by chloroform. It was not obtained in crystalline form and was probably racemized, but the alkali racemization does not alter the properties investigated in this paper. No violin (cf. Watson, 1932) was found in this urine.

For the investigation of the clinical samples a 24 hours specimen of the patient's urine was collected in a dark-glass bottle under petrol-ether. After acidification with acetic acid urobilinogen was extracted with petrol-ether. It was oxidized to urobilin by allowing the petrol-ether solution to stand in bright sunlight for 12 hours. Urobilin was extracted by dilute sodium carbonate solution and it was extracted from the acidified aqueous solution by chloroform (urobilinogen fraction). The urobilin remaining in the urine after petrol-ether extraction was brought into chloroform by ammonium sulphate (urobilin fraction). A sample of the latter solution was tested for admixed pigments by extracting the urobilin with 25 p.c. hydrochloric acid. Usually the total pigment was removed from the chloroform layer. If this did not happen, the chloroform solution was extracted by 25 p.c. hydrochloric acid and the pigment was brought back into fresh chloroform after dilution of the acid solution.

*Choletelin.*[6] This urobilinoid pigment, a higher oxidation product of mesobiliverdin, was obtained by two methods:

(a) To a solution of mesobiliverdin-zinc complex salt in methyl alcohol N/10 alcoholic iodine solution was gradually added, until the red fluorescence of mesobiliviolin-zinc, appearing at first, had been displaced by the green fluorescence of choletelin-zinc, and the absorption band of the former in the orange by the band of the latter in the blue-green. After 8 atoms of iodine per molecule of mesobiliverdin had been added, the reaction was almost complete, and the violin-zinc bands had entirely disappeared after two more atoms of iodine had been added. The solution was diluted with water and acidified with hydrochloric acid. Choletelin was extracted by chloroform.

(b) To a solution of mesobiliverdin in methyl alcohol half the volume of dilute aqueous hydrochloric acid was added and then gradually a strong solution of sodium nitrite. The solution was kept acid to congo red by occasional additions of hydrochloric acid and the reaction continued until the absorption bands of acid mesobiliviolin (598 mμ and 556 mμ), appearing at first, had been replaced by a band in the blue-green. The choletelin was extracted by chloroform.

*Mesobilinogen* was prepared by reduction of bilirubin with sodium amalgam in the customary way. "*Körper II*" is an amorphous urobilinoid by-product of this preparation. *Urobilinogen* was obtained in the same way from urobilin; other samples were directly extracted by petrol-ether from urines acidified with acetic acid.

*Stercobilinogen* was obtained from stercobilin by amalgam reduction or directly from faeces by the method of Terwen (1925). The various preparations of urobilinogen (with a few exceptions, cf. Table 2) and of stercobilinogen showed no differences.

### *Absorption Spectra of Urobilin and Urobilin-IXα.*

Lemberg (1934) found a difference of 23Å in the position of the absorption bands of urobilin and urobilin-IXα hydrochlorides in acid methyl alcoholic solutions. Without mentioning this publication Watson as well as Siedel repeatedly stated that they found no difference between the absorption spectra of the two compounds. We bring now further evidence for the existence of this difference.

The pigments were dissolved in methyl alcohol with the addition of a drop of 10 p.c. hydrochloric acid, and the position of the absorption bands was measured

[6] Strictly its name ought to be "mesocholetelin", since a similar substance can be obtained from biliverdin.

with the Hartridge reversion spectroscope. It is essential to use greatly diluted solutions of a concentration in which the band is of minimum breadth, though still strong enough to be measured exactly (about 0·5 mg. in 100 c.c.). Table 1 gives the positions of the absorption bands in Å. The number in brackets behind the description of the preparation on the top of the columns is the number of spectroscope readings taken for each of the preparations, and the number behind the position in Å indicates the standard error of this mean value in Å. For the measurements in 1937 five different preparations each of stercobilin and of urobilin-IX$\alpha$ were used, and for each preparation 10 readings were obtained on each of two successive days. The lowest row gives the difference in Å found between the positions of the absorption bands of the two compounds.

TABLE 1.

| | Crystalline products (30) | Amorphous products in 1937 (100) | Amorphous products in 1934 (20) | |
|---|---|---|---|---|
| Stercobilin | 4,920 ± 1 | 4,919 ± 0·4 | 4,912 ± 1·5 | 4,912 ± 1·5 |
| Urobilin (Urinary) | | | | 4,915 ± 1 |
| Urobilin-IX$\alpha$ | 4,940 ± 1·5 | 4,938 ± 0·7 | 4,937 ± 1·5 | |
| $\Delta$ | 20 | 19 | 25 | 3 |

While the bands of faecal urobilin and of urinary urobilin (from Banti's disease) differ by only 3Å, those of the former and of urobilin-IX$\alpha$ differ by about 20Å. The probability of this difference being accidental is $< 10^{-9}$ for the 1937 measurements and $< 10^{-7}$ for the 1934 experiments. The difference between urinary and faecal urobilin is not statistically significant, but it may be caused by a slight amount of urobilin-IX$\alpha$ being present in the urobilin from this urine.

The absorption band of choletelin was found to lie at 492·5–492·8m$\mu$, i.e. between those of urobilin and urobilin-IX$\alpha$. The band is somewhat less defined than those of the other pigments. The absorption band of "Körper II" was undistinguishable from that of urobilin-IX$\alpha$.

The results were confirmed by the study of the absorption curves by both the subjective and the photographic spectrophotometric method.

In the Hilger visible spectrophotometer the maxima of the bands in 3 p.c. alcoholic hydrochloric acid were found at 4,900 Å for urobilin and at 4,925 Å for urobilin-IX$\alpha$. The absorption curves of the two substances in the same solvent measured in the Echelon Cell with a Hilger U. V. spectrophotometer were shown in fig. 1. The curves have been constructed from two plates (Wellington Antiscreen) each giving 2 exposures of the solution of one substance (Plate 1a: 25·3 mg. urobilin in 100 c.c., D (sector) = 1·0. Exposure: 3 mins. 1b: the same, D = 0·6, 2 mins. 2a: 23 mg. urobilin-IX$\alpha$ in 100 c.c. D = 1·0, 3 mins. 2b: the same. D = 0·6, 2 mins.). The curves show the specific extinction coefficient ($\epsilon = 1/\text{cxd. log } I/I_0$, where I and $I_0$ are transmitted and incident light intensities, c the concentration of the solution in gm. per l and d the length of the layer in cm.) plotted against wave lengths. The absorption maximum of urobilin was

found at 4,870 Å, that of urobilin-IX$\alpha$ at 4,900 Å. At a level of $\epsilon = 18 - 20$ the difference in the position of the two bands is much less and this may explain why the other authors did not observe it.

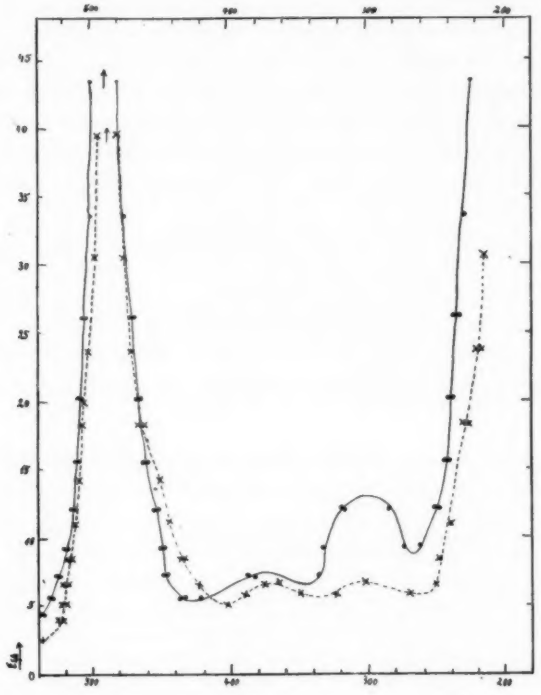


**Fig. 1.**

———— Urobilin-IX$\alpha$.
- - - - - - - Urobilin (= Stercobilin).

In the ultra-violet part of the spectrum urobilin has a second band at 3650Å and a third at about 3000Å, while the bands of urobilin-IX$\alpha$ lie at 3750Å and 3070Å, the latter being stronger than the corresponding band of urobilin. All these bands are weak and ill-defined.

The position of the absorption bands of the zinc complex salts of the urobilins varies too much with the conditions to be used for a distinction between the pigments. In slightly acid solution the band of urobilin-IX$\alpha$-zinc salt seems also to lie somewhat more towards the red (5090Å) than that of urobilin (5070Å), but in ammoniacal solution no such difference exists (both bands 5060Å).

*Other Differences Between Urobilin and Urobilin-IXα.*

Hopkins and Garrod observed the following differences between urobilin and hydrobilirubin:

(1) An acid solution of urobilin is yellow, however strongly it is diluted, while a very dilute solution of hydrobilirubin is of a reddish colour.

(2) When a solution of urobilin in sodium bicarbonate is acidified by a slight excess of dilute sulphuric acid, the solution displays an absorption band at about 5300Å ("E" band), while hydrobilirubin does not show the phenomenon.

(3) Urobilin is much more stable than hydrobilirubin.

Siedel (1936) did not observe the "E" band with urobilin, and found it once with urobilin-IXα.

We can confirm all three results of Hopkins and Garrod. Pure crystalline urobilin-IXα displays a reddish colour in very dilute acid solution which is quite different from that of a urobilin solution of the same concentration. The "E" band and a green fluorescence (or pseudo-fluorescence) was regularly obtained with urobilin, but not with urobilin-IXα. On standing in acid methyl alcohol a solution of urobilin remains unaltered, while a solution of urobilin-IXα turns violet.

*Ferric Chloride Method for Distinguishing Between Urobilin and Urobilin-IXα.*

The sample of urobilin is dissolved in 2 c.c. of methyl alcohol in a test tube and 1 c.c. of a solution of ferric chloride (0·2 g.) in conc. hydrochloric acid is added. The test tube is placed into a beaker with boiling water and the reaction mixture is kept boiling for 15 minutes. When the solution is poured into a mixture of 8 c.c. of strong sodium acetate solution and 8 c.c. of ether and this is shaken in a separating funnel, the ethereal solution takes up the oxidation products of urobilin-IXα, while the greater part of the unaltered urobilin remains in the aqueous layer. Subsequent washing of the ether with water (4 times) removes urobilin, but leaves the blue oxidation products of urobilin-IXα in the ether. Four extractions with 2 c.c. each of 10 p.c. hydrochloric acid extract these from the ether. 0·05 mg. of urobilin-IXα cause a distinct blue colour of the HCl extract and 0·5 mg. a deep blue, while 1 mg. of stercobilin gives only a weak red-violet colour. The intensity of the blue colour of the HCl extract is well graded with the amount of urobilin-IXα used. The amount of urobilin-IXα can thus be determined in a urobilin containing only a small fraction of urobilin-IXα besides urobilin. If the total amount of urobilin is known the proportion of urobilin and urobilin-IXα in the sample can be determined.

Until now we have applied this quantitative method only for the determination of the proportion of the two pigments in normal urine. The amount of total urobilin was determined by a spectrocolorimetric comparison of the solution in methyl alcohol containing 1 p.c. HCl with a standard of urobilin in the same solvent. This method can be applied only when there is a small proportion of urobilin-IXα present, since the strength of the absorption bands of the two compounds is different. (In other instances it would be necessary to establish at first roughly the proportion of the two pigments as done here, and then to measure again the total urobilin concentration against a standard of a mixture of urobilin and urobilin-IXα in this proportion.) A solution of 1 mg. from normal urine in 2 c.c. methyl alcohol was subjected to the ferric chloride oxidation, and

the colour of the acid extract compared with the colour of extracts obtained from the same amount of a urobilin mixture with a varying proportion (0, 10 p.c., 20 p.c.) of urobilin-IX$\alpha$. The extract obtained from normal urinary urobilin was weak red-violet, somewhat stronger coloured than the weak red-violet extract from pure urobilin, but distinctly different from the blue-violet extract obtained from the mixture of 0·9 g. urobilin and 0·1 mg. urobilin-IX$\alpha$. Urobilin from normal urine may, therefore, contain a slight amount of urobilin-IX$\alpha$, but certainly less than 10 p.c. In agreement with this finding no difference was found spectroscopically in the position of the absorption bands of pure urobilin and of normal urinary urobilin.

Using the ferric chloride method we convinced ourselves that mesobilinogen when subjected to the treatment used for the isolation of urobilin from urine or from faeces, is not transformed into urobilin, nor can urobilinogen be obtained from mesobilinogen by protracted reduction with sodium amalgam. The characteristic differences between urobilin and urobilin-IX$\alpha$ in acid absorption spectrum and ferric chloride oxidation do not disappear by transformation of the compounds into their zinc complex salts and subsequent recovery by acid, or by dissolving in alkali and reacidification, reactions in which the optical activity of urobilin is destroyed.

Choletelin is not oxidized by ferric chloride to blue products, which is to be expected, since it arises from mesobiliverdin by oxidation. When choletelin is reduced by sodium amalgam a chromogen is obtained in a rather poor yield which reacts in the ferric chloride reaction like mesobilinogen and is probably identical with it. Choletelin can thus be distinguished from urobilin as well as from urobilin-IX$\alpha$. The formula ascribed to urobilin by Lemberg in 1934 may be correct for choletelin.

*Investigation of Pathological Urines.*

The method of isolation of urobilin from these urines has been described above. The results are collected in Table 2. The third column gives the position of the absorption maximum in acid methyl alcoholic solution in m$\mu$ as the mean of ten readings. Where two figures are given the first refers to the urobilinogen fraction, the second to the urobilin fraction.

The results can be divided into four classes (I-IV in the last column) according to the position of the absorption band. Of course, such a division is rather arbitrary. Statistically the proportion of urobilin-IX$\alpha$ in a mixture with urobilin can be estimated by ten readings satisfactorily with an accuracy of only $\pm$ 20 p.c. ($0{\cdot}05>P>0{\cdot}02$, i.e. 2–5 p.c. of the values overlapping), while a distinction of a difference of 10 p.c. with 25 p.c. overlapping is not satisfactory. Of the 40 urines investigated 23 belong to class I, in which the centre of the absorption band was found at a wave-length smaller than 492·4 m$\mu$ with an average of 492·1m$\mu$. From this we can conclude that in the majority of pathological urines urobilin-IX$\alpha$ is either not present or forms less than 20 p.c. of the total urobilin. In class II (10

TABLE 2.

| Patient. | Disease. | Schlesinger Test. | Position of urobilin band in urobilinogen fraction. | | in urobilin fraction. | | |
|---|---|---|---|---|---|---|---|
| J.D. | pernicious anaemia | + | | 491·9 | | | I |
| W.McI. | cardiac failure, senility | — | | 492·8 | | | II |
| A.Mc. | hypochromic anaemia | — | 492·3 | | 491·9 | | I |
| W.M. | cardiac failure | — | | 492·1 | | | I |
| G.P. | cholelithiasis and empyema of gall bladder, moribund | ++ | | 491·5 | | | I |
| N.R. | acute phlebitis and scoliosis | — | 492·6 | | 492·7 | | II |
| M.St. | peritoneal adhesions | — | | 493·5 | | | III |
| A.M. | cholelithiasis | + | 492·2 | | 492·4 | | I |
| H.F. | pernicious anaemia | + | | 493·2 | | | III |
| E.C. | cholelithiasis, chronic cholecystitis | ++ | | 493·0 | | | III |
| T.G. | arsenic poisoning | (+) | 491·8 | | 492·3 | | I |
| X.A. | liver cirrhosis; potator | ++ | 492·9 | | 492·1 | | II |
| R.H. | haematuria, dysuria | — | | 492·2 | | | I |
| F.B. | cardiac failure | + | 492·2 | | 492·5 | | I |
| E.E. | cholecystitus | ++ | | 492·6 | | | II |
| A.R. | pancreatitis | (+) | | 492·1 | | | I |
| M.F. | chronic haemolytic anaemia | + | | 492·5 | | | II |
| R.A. | rheumatic arthritis | — | | 491·8 | | | I |
| M.T. | jaundice | ++ | | 492·2 | | | I |
| X.O. | liver cirrhosis | + | | 494·0 | | | IV |
| B.X. | liver carcinoma | + | | 492·4 | | | I |
| I.H. | subacute rheumatism | — | | 492·1 | | | I |
| I.E. | depressive psychosis with digestive symptoms? | — | 492·9 | | 492·4 | | II |
| E.G. | chronic cholecystitis | — | 492·5 | | 491·3 | | I |
| I.A. | infected finger | — | 492·6 | | 492·0 | | I |
| L.F. | acute cholelithiasis | — | 492·2 | | 493·2 | | II |
| A.S. | carcinoma recti | ? | | 492·9 | | | II |
| B.S. | liver cirrhosis, ascites | — | 494·3 | | 494·5 | | IV ferric chloride reaction positive |
| H.W. | chronic gastritis, megalocolon, melaena | — | 493·4 | | 492·8 | | III |
| M.W.Th. | toxaemia of pregnancy, secondary haemorrhage | — | 493·4 | | 494·0 | | IV |
| St.S. | coronary occlusion | — | | 492·8 | | | II |
| A.F. | carcinoma of colon? | + | | 491·9 | | | I |
| A.B. | cardiac failure | — | | 491·8 | | | I |
| M.Wh. | purpura haemorrhagica | — | | 491·9 | | | I |
| S.D. | gastric ulcer, pyloric stenosis, adhesions | — | 493·2 | | 492·2 | | II |
| X.M. | cardiac failure | — | | 491·3 | | | I |
| L.R. | oesophageal orifice hernia, multiple diverculitis of intestine | — | 492·8 | | 492·2 | | I |
| F.S. | jaundice | — | | 492·4 | | | I |
| X.F. | hyperchromic anaemia | + | | 492·1 | | | I |
| X.M. | malignant gall bladder | + | | 492·1 | | | I ferric chloride reaction negative |

urines) the band was found to lie between 492·5 and 492·9 mμ with an average of 492·7mμ, corresponding to a probable content of 25–45 p.c. urobilin-IXα. In class III (4 urines) the band was found at 493·0–493·5mμ with an average of 493·2mμ; urobilin-IXα forms here the somewhat greater part of the total urobilin (50–75 p.c.). Only in three cases was the band that of urobilin-IXα. Two of these last-mentioned urines were from patients with liver cirrhosis, one from a patient with toxaemia of pregnancy. In the patients with urines containing much urobilin-IXα we find patients with chronic cholecystitis, pernicious anaemia, chronic gastritis with melaena, and peritoneal adhesions (this patient had a gall bladder operation six years ago). At present it is not yet possible to draw conclusions concerning the significance of urobilin-IXα as a pathological urobilin or concerning its formation in the body. Urobilin-IXα predominated in two cases of liver cirrhosis, but in a third case (X.A.) comparatively little of it was present. While one pernicious anaemia urine had a considerable proportion of urobilin-IXα, none was present in a second urine of a patient with this disease. Similar observations were made in chronic cholecystitis. The data of Table 2 show clearly that urines rich in urobilin may not contain any urobilin-IXα and that urines without considerably increased urobilin content may contain urobilin-IXα predominantly. Of 17 urines containing abnormally large amounts of urobilin 11 are of class I, 3 of class II, 2 of class III, and only one contains urobilin-IXα alone.

A certain reserve is advised, since the danger of impurities shifting the position of the absorption band of urobilin cannot be quite excluded. In order to avoid the great loss of pigment unavoidably connected with rigorous purification, the crude urobilin extracts have been applied. We do not consider this danger as great, because the absorption band of urobilin is well marked. Even in normal urine, in which the proportion of the impurities to urobilin is by far the greatest, we have been unable to find evidence for a shift of the absorption maximum by the impurities, and no discrepancy has been found between the results of the spectroscopic method and of the ferric chloride oxidation method. In two cases in which the ferric chloride test was applied qualitatively for pathological urines, its result agreed with that of the spectroscopic method. Nevertheless, a more extensive application of the ferric chloride method is indicated. There can be little doubt, however, that the majority of pathological urines do not contain a great proportion of urobilin-IXα, if they contain it at all, and that urines in which urobilin-IXα predominates are rather rare.

## ACKNOWLEDGMENTS.

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## SUMMARY.

Methods are evolved to distinguish urobilin (stercobilin) or urobilinogen (stercobilinogen) from urobilin-IX*a* or mesobilinogen and to determine their amounts in semi-quantitative micro-tests. Use is made of the different situation of the absorption bands of urobilin and of urobilin-IX*a* in acid alcoholic solutions and of their behaviour towards ferric chloride.

The ultra-violet absorption spectra of the two urobilins are described.

In contradistinction to urobilin-IX*a* urobilin gives the "E" band found by Garrod and Hopkins.

The urobilinoid substance choletelin, formed by oxidation of mesobiliverdin, can be distinguished from urobilin-IX*a* by its resistance to oxidation and from urobilin by sodium amalgam reduction leading to mesobilinogen.

While some pathological urines, particularly of patients with damaged livers, contain urobilin-IX*a* admixed to urobilin and (rarely) even predominant, urobilin (stercobolin) forms at least 90 p.c. of the total urobilins of normal urine and at least 80 p.c. of the total urobilins in the majority of pathological urines. It is, therefore, suggested to retain Jaffé's name "urobilin" for the normal urinary urobilin, now called "stercobilin" by Fischer and his school. The assumption that mesobilinogen is the mother substance of urobilin has no sound basis.


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# STUDIES ON HAEMOLYTIC STREPTOCOCCI FROM HUMAN SOURCES

## I. THE CULTURAL CHARACTERISTICS OF POTENTIALLY VIRULENT STRAINS.

by

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Some of the biological characteristics of haemolytic streptococci from human sources have been described by Ward and Lyons (1935). Virulent strains were found in many cases to have a distinctive colony form, but subsequent experience has shown that although virulence is commonly associated with two well-marked types of colony, there are other colonial forms which are difficult to interpret.

It is known that haemolytic streptococci belonging to the majority of the serological Groups of Lancefield (1933) are often found in the human subject, but evidence has accumulated in the last few years that human infections with the haemolytic streptococcus are almost always caused by organisms belonging to Group A. If, then, the serological Group of a haemolytic streptococcus can be determined, it can be assumed that it is potentially virulent for human beings if it belongs to Group A, and that it is presumably non-virulent if it belongs to any of the other Groups. Since there is no reliable animal test for virulence, the value of determining the serological Group is evident. The method of carrying out the Lancefield precipitin test for determining the Group to which a haemolytic streptococcus belongs is straightforward and the results are sharp and easily read. Its only drawback is that it takes time to carry it out properly, too much time for the test to be a very practicable one in a busy routine laboratory. In laboratories where facilities are not available for carrying out the precipitin test, the mere finding of the haemolytic streptococcus in the throat of an individual is usually taken as evidence that he or she is a potentially dangerous carrier. This is not necessarily the case, and if there were available an easier test which could be used to indicate whether the organism were potentially virulent, this information would be of value in the investigation, for example, of the source of infection in puerperal sepsis.

It is the purpose of this paper to describe a simple test which is suitable for

use in an ordinary routine laboratory and which, with very few exceptions, distinguishes the pathogenic from the non-pathogenic haemolytic streptococci from human sources. It depends on the growth characteristics of these organisms in two special media. There is good correlation between the information afforded by this biological test and that afforded by Lancefield's serological test. No claim is made that the cultural test is better than the precipitin test in assessing the potential virulence of haemolytic streptococci for human beings, but merely that it is more suitable for use in a routine laboratory.

The growth characteristics on which this test depends are themselves of some interest, and are dealt with in the discussion.

## EXPERIMENTAL.

### SOURCE OF MATERIAL:

1. All of Griffith's (1934) type strains sent to us by Dr. Griffith.
2. Strains from various human infections outside the nasopharynx.
3. Strains from the nasopharynx of normal individuals, of patients suffering from tonsillitis, scarlet fever and diphtheria.

### TECHNIQUE:

1. *Isolation of strains:*

Throat swabs or specimens of pus were plated out on ordinary horse-blood agar plates. In some cases we experienced difficulty in deciding whether or not an organism was a true haemolytic streptococcus from its appearance on the ordinary horse-blood agar plates. Later we found that partial haemolysis was associated with a particular variant of the haemolytic streptococcus which is described below. This difficulty can be minimized by using a special horse-blood agar plate which is prepared in the following way: In 1 litre of water are dissolved 10 g. of neopeptone, 8·5 g. of sodium chloride and 0·5 g. of glucose. The pH is adjusted to 7·6, 15 g. of agar powder are added and dissolved by boiling. The medium is then distributed in 14 c.c. quantities in 6 in. × ¾ in. tubes, sterilized in the autoclave and stored in the ice box. When a blood plate is required, a tube of neopeptone agar is melted, cooled to 50°C. and 1·5 c.c. of horse serum and 1·0 c.c. of horse blood are added, mixed, and the plate is poured. These blood plates can be stored in the ice box for a day or two before use, but not longer. They are soft and must be spread carefully with a fine gauge platinum wire. Despite these inconveniences, we definitely prefer this special medium to the ordinary horse-blood agar, because colonies of haemolytic streptococci are much easier to recognize on it, and also because it shows up the colony differences much more sharply. If more agar is added to the medium, or if the horse serum is more than six weeks old the colonial differentiation is apt to be masked.

If it is necessary to preserve the organism, a colony is picked off into a tube of Robertson's cooked meat medium (with a layer of paraffin oil), the tube is incubated for twenty-four hours and stored in the ice box. Cultures in the meat medium remain viable for at least eighteen months.

2. *Appearance of colonies on the special neopeptone horse-blood agar plates.*

There are two very distinctive colonies which cannot be mistaken:

(a) A colony which under the colony microscope (20 × magnification) appears like the top of a molar tooth when examined by reflected light. By transmitted light, the colony shows an irregular opaque centre embedded in a transparent matrix. With the naked eye or the hand lens these colonies appear rather like flecks of dust on the surface of the medium. The haemolysed

area surrounding these colonies is apt to be narrow, and in this event the typical colony form is of great help in deciding whether the organism is a haemolytic streptococcus. This difficulty is often accentuated on an ordinary horse-blood agar plate and is one of the reasons why we prefer the special plate when working with a mixed flora. In unpublished experiments, Dr. N. E. Goldsworthy found that this particular variant when grown in serum broth only produced about one-fourth as much haemolysis as other variants of the haemolytic streptococcus. This is the "F" variant of Ward and Lyons (1935) and the "rough coherent" colony of Griffith (1934).

(b) A colony, which, under the colony microscope presents, when young, the appearance of a smooth, watery glistening dome. As the culture ages, the centre of the colony falls in and the colony flattens out, often showing a rough, pock-marked surface. With the naked eye or the hand lens, the colony is recognized by its large size, and its flatness. This is the "M" variant of Ward and Lyons (1935) and the watery, mucinous colony described by Griffith (1934) and other authors.

The other colony forms observed on the special medium vary, the commonest being conical in shape, but sometimes umbonate colonies are met with. We do not feel confident in differentiating one from another.

3. *Special media for detecting the growth characteristics of the organisms.*

(a) Serum broth (S.B.). This medium consists of a mixture of three parts horse serum and one part tryptic digest broth. The tryptic digest broth is prepared in the following way which is slightly modified from Pauli and Coburn (1937): Take 400 g. of beef heart (all fat removed), 0·5 g. of disodium phosphate and 1 litre of water and adjust to pH 9 (approximately) with N sodium hydroxide. Heat at 70°–80° C. for five minutes. Cool to 45°C. and add 2·0 g. of trypsin (B.D.H.) suspended in 10 c.c. of saline. Incubate for 45 minutes in a 45°C. water-bath. Adjust to pH 6·0 (approximately) with N hydrochloric acid and boil for 20 minutes. Filter through paper and when cool adjust to pH 7·8. Add calcium chloride to a concentration of 0·13 p.c. and sodium chloride to a concentration of 0·25 p.c. Boil for five minutes and filter through paper. Sterilize in the Arnold at 100°C. on three successive days.

The mixture of horse serum and tryptic digest broth is distributed with a sterile pipette in 2·0 c.c. amounts into 3½ in. × ½ in. test tubes and stored in the ice box.

(b) Serum peptone agar (S.P.A.). This medium consists of a mixture of diluted horse serum and neopeptone solution containing a low percentage of agar. It is prepared in the following way: (i) Dissolve 0·4 g. of agar powder, 1·0 g. of neopeptone, 0·85 g. of NaCl in 100 c.c. of distilled water, adjust pH to 7·6, filter through paper, and sterilize in the autoclave in Flask A. (ii) Into Flask B measure 75 c.c. of saline solution and 25 c.c. of horse serum (not more than eight weeks old), which has been heated to 56°C. for thirty minutes.

Melt the contents of Flask A and after cooling to 50–55°C., add to the contents of Flask B which has been warmed to 50–55°C. Mix and distribute the mixture with a sterile pipette in 3·0 c.c. amounts into sterile 3½ in. × ½ in. test tubes. The tubes are stored in the ice box and in any case should be cooled down in the ice box before use.

4. *Inoculation of special media.*

(a) From colonies on a special blood plate or on an ordinary blood plate: the colony is touched with a straight platinum wire which is rubbed on the glass at the top of the fluid in one of the tubes of S.B. The tube is then rotated rapidly three or four times between the hands. One loopful from the S.B. tube is inoculated into the S.P.A. tube and the contents of the latter tube are mixed by rotating it rapidly between the hands.

(b) From broth cultures: a loopful of the broth culture is inoculated into a tube of S.B. and the contents mixed; a loopful from the S.B. tube is inoculated into the S.P.A. tube and the contents mixed.

Both tubes are then incubated for twenty-four hours at 37°C.

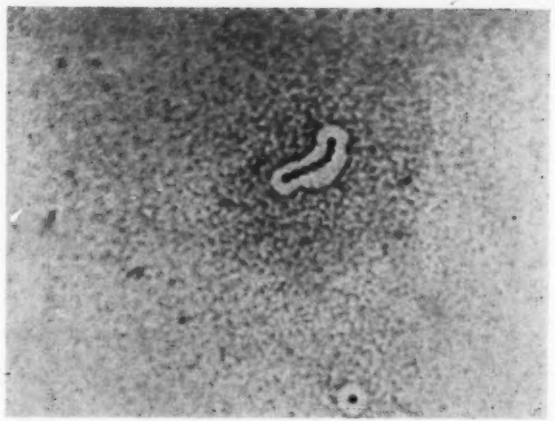

**Fig. 1.** India-ink preparation of Group A capsulated haemolytic streptococci showing wide clear halo round organisms.

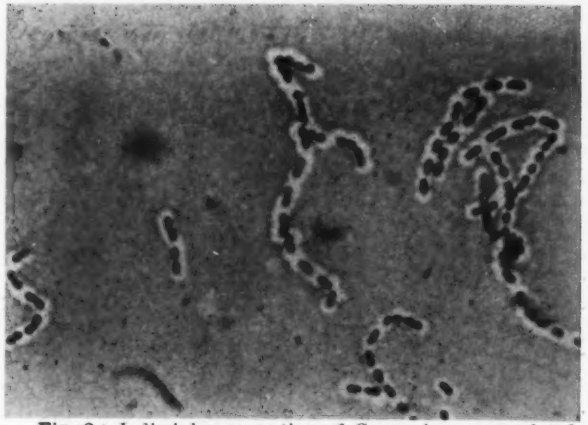

**Fig. 2.** India-ink preparation of Group A non-capsulated haemolytic streptococci, showing narrow clear halo round organisms.

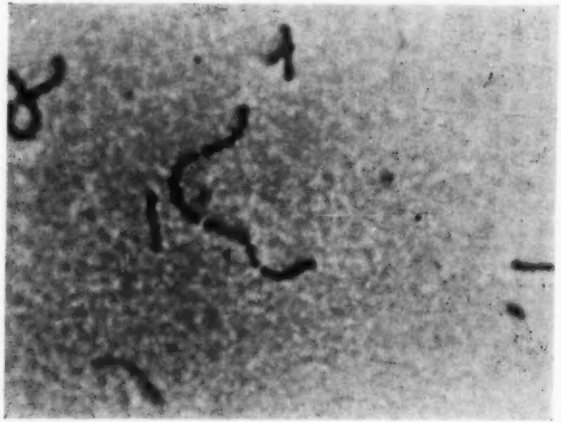

**Fig. 3.** India-ink preparation of non-capsulated haemolytic streptococci not belonging to Group A, showing ink coming right up to the chains.

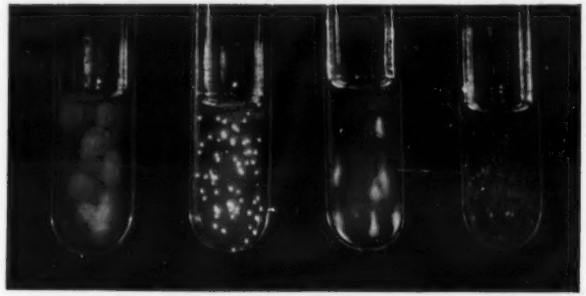

**Fig. 4.** Tube 1: Group A capsulated haemolytic streptococci growing as feathery colonies in serum peptone agar. Tube 2: Group A non-capsulated haemolytic streptococci growing as compact opaque colonies in serum peptone agar. Tube 3: Type 1 Pneumococcus growing in normal horse serum peptone agar; feathery colonies. Tube 4: Type 1 Pneumococcus growing in Type 1 antiserum peptone agar; compact colonies.

5. *Observation of growth characteristics in the special media.*

After incubation the appearances of the growth in the two tubes are studied.

(a) The S.B. tube. Here the growth may be either diffuse of flocculent. The non-capsulated organisms grow flocculently and the capsulated streptococci[1] as a rule grow diffusely but not always. Certain strains produce a marked opalescence (Fig. 6) in this medium. If the strain be one that grows diffusely, the opalescence may be masked to a certain extent, but may be detected easily enough by looking down at the tube against a dark background.

**Fig. 5.** Suspended colonies in a slightly modified weak agar medium, consisting of equal parts of undiluted horse serum and neopeptone agar (0·4 p.c.). Tube 1: Group A non-capsulated haemolytic streptococci. Compact colonies surrounded by a zone of denatured serum protein. Tubes 2 and 3: Group A capsulated haemolytic streptococci; feathery colonies. Tube 4: Non-capsulated haemolytic streptococci not belonging to Group A: compact colonies. Tube 5. Type 1 pneumococcus; feathery colonies. Tube 6: Rough avirulent pneumococcus; compact colonies.

(b) The S.P.A. tube. There is enough agar in this medium to keep the organisms suspended, but not enough to restrict their outgrowth into the medium. The capsulated streptococci form semi-transparent feathery colonies (Figs. 4 and 5), sometimes spherical, sometimes elongated, sometimes shaped like a medusa-head. In some cases a nucleus of denser growth may be observed in these colonies.

The non-capsulated streptococci form opaque compact colonies (Figs. 4 and 5), sometimes star-shaped, easily distinguished from the feathery colonies of the capsulated strains.

If the colonies in the S.P.A. tube are too crowded, they are unable to develop their characteristic form. There should not be more than about fifty suspended colonies in a tube.

6. *India-ink preparations.*

Following the technique of Butt, Bonynge and Joyce (1935) we have confirmed their claims that 6 p.c. glucose as a suspending fluid gives much clearer pictures than saline. A loopful of 6 p.c. glucose is placed on the end of a slide, a loopful of culture and a loopful of Gurr's india-ink are mixed with the glucose and a thin film made in the same manner as a blood film. The films are allowed to dry in the air and stained for one minute with alkaline methylene blue. The stain is washed off with water and the film allowed to dry without blotting. The stained film is then examined under the microscope.

[1] It is perhaps not generally accepted that a large number of strains of haemolytic streptococci belonging to Group A are capsulated. Nevertheless it can be shown clearly and definitely that there is a stained capsular structure surrounding young organisms of such strains if the technique described in the text of this paper is carried out. Capsulation of streptococci was described by Bordet (1897) many years ago and in recent years has been studied by Seastone (1934) and by Ward and Lyons (1935).

In the case of the haemolytic streptococci, the picture in these preparations is conditioned by the age of the culture, the nature of the medium and the strain of the organism. The clearest differentiation is obtained by the use of a culture grown for four hours in equal parts of horse serum and 1 p.c. neopeptone water (pH 7·3). Here the capsulated streptococci show a wide, clear halo surrounding the chains (Fig. 1). Some of the non-capsulated streptococci show a narrower halo (Fig. 2), while with other strains the ink comes right up to the cocci (Fig. 3). In older cultures the haloes tend to disappear.

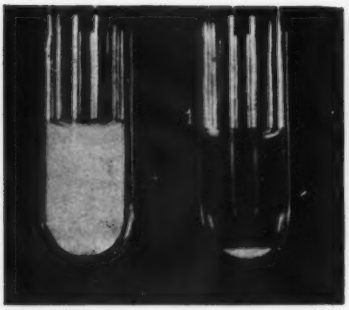

**Fig. 6.** Tube 1: Group A non-capsulated haemolytic streptococci growing in serum broth showing the opalescent supernatant fluid above the deposit of organisms. Tube 2: Non-capsulated haemolytic streptococci not belonging to Group A, showing clear supernatant fluid above deposit of organisms.

**Fig. 7.** Tube 1: "Smooth" Shiga dysentery bacilli growing in serum peptone agar as feathery colonies. Tube 2: Virulent non-motile typhoid bacilli growing in serum peptone agar as feathery colonies. Tube 3: Typhoid bacillus 0901 growing in serum peptone agar as compact colonies with small feathery undergrowths.

7. *Capsules.*

A drop of a four-hour culture in 50 p.c. horse serum peptone water is added to 0·25 c.c. of defibrinated human blood and incubated at 37°C. for thirty minutes. A thin film of the blood is made and allowed to dry in air. The slide is covered with about 2 c.c. of Leishman's stain and after six minutes an equal quantity of distilled water is added and allowed to remain on the slide for four minutes. The diluted strain is washed off with water and the film allowed to dry without blotting. Capsules appear as a definite pink-staining structure surrounding the black cocci.

8. *Lancefield's Group A precipitin test.*

This is carried out in the manner described by Lancefield (1933).

## RESULTS.

### *Detailed Description of Type Strains.*

In Table 1 will be found the results obtained by examining all the Griffith Type cultures by the methods described above. It must be recognized that the actual culture which we have described in Table 1 may be one of two or three

variants all belonging to the particular serological Type. For example, in Type 1, Griffith (1934) described three variants:

(a) a rough coherent colony ("F" in our nomenclature);
(b) a watery mucinous colony ("M" in our nomenclature); and
(c) soft and translucent.

TABLE 1.

*Serological Grouping and Biological Characteristics of Griffith's Type Cultures.*

| Organism | "A" group precipitin test. | Colony form on special blood plate. | Appearance of growth in serum broth. | Opalescence in serum broth ("S.B."). | Appearance of colony in serum peptone agar ("S.P.A."). | Haloes in india-ink preparation. | Capsules. |
|---|---|---|---|---|---|---|---|
| Type 1 | + | Conical | Diffuse | 0 | Feathery | + | + |
| Type 2 | + | Conical | Diffuse | + | Feathery | + | + |
| Type 3 | + | Conical | Diffuse | 0 | Feathery | + | + |
| Type 4 | + | Conical | Flocculent | + | Compact | 0 | 0 |
| Type 5 | + | Conical | Diffuse | 0 | Feathery | + | + |
| Type 6 | + | M | Diffuse | 0 | Feathery | + | + |
| Type 7 | 0 | Conical | Flocculent | 0 | Compact | 0 | 0 |
| Type 8 | + | F | Flocculent | + | Compact | + | 0 |
| Type 9 | + | Conical | Diffuse | + | Feathery | + | + |
| Type 10 | + | Conical | Diffuse | 0 | Feathery | + | + |
| Type 11 | + | Conical | Diffuse | 0 | Feathery | + | + |
| Type 12 | + | Conical | Flocculent | 0 | Compact | 0 | 0 |
| Type 13 | + | M | Diffuse | + | Feathery | + | + |
| Type 14 | + | M | Diffuse | 0 | Feathery | + | + |
| Type 15 | + | Conical | Diffuse | 0 | Feathery | + | + |
| Type 16 | 0 | F | Flocculent | 0 | Compact | 0 | 0 |
| Type 17 | + | M | Diffuse | 0 | Feathery | + | + |
| Type 18 | + | M | Diffuse | 0 | Feathery | + | + |
| Type 19 | + | M | Diffuse | 0 | Feathery | + | + |
| Type 20 | 0 | Conical | Flocculent | 0 | Compact with short down-growth | 0 | 0 |
| Type 21 | 0 | Conical | Flocculent | 0 | Compact | 0 | 0 |
| Type 22 | + | F | Flocculent | + | Compact | + | 0 |
| Type 23 | + | M | Diffuse | 0 | Feathery | + | + |
| Type 24 | + | M | Diffuse | 0 | Feathery | + | + |
| Type 25 | + | F | Flocculent | + | Compact | + | 0 |
| Type 27 | + | F | Flocculent | + | Compact | + | 0 |
| Type 28 | + | F | Flocculent | + | Compact with feathery outgrowth | + | +* 0 |
| Type "Coggins" | + | M | Diffuse | 0 | Feathery | + | + |
| Type "Quinn" | + | M | Diffuse | 0 | Feathery | + | + |
| Type "Roumania" | 0 | Conical | Flocculent | 0 | Compact | 0 | 0 |

* This strain appears to be intermediate between the non-capsulated "F" and the capsulated "M" form. We have met several other strains among our own isolations which behaved in this manner.

The last appears to be the actual colony form of Type 1, which we have described in our table. In other words, our findings for each Type are only true for

the particular variant belonging to that Type, which we have studied. Nevertheless, certain generalizations may be made from these results which can be stated as follows.

*Generalization from Study of Type Strains.*

1. The capsulated organisms belonging to Group A may either grow as a typical mucous ("M") colony or show colony forms which are conical or umbonate. They grow diffusely in S.B. and a few types produce opalescence in this medium. They form a feathery colony in the S.P.A. medium, and young cultures have a wide halo in the india-ink preparation.

2. The non-capsulated organisms belonging to Group A nearly always have an "F" colony, they grow flocculently in S.B. with pronounced opalescence, while in the S.P.A. medium the colony is compact and opaque. Young cultures in serum-peptone show a definite but narrowed halo than the capsulated strains.

3. The Type cultures which do not belong to Group A are Type 7 (Group C), Type 16 (Group G), Type 20 (Group C), Type 21 (Group C), and Type Roumania (? Group). These are non-capsulated organisms which grow flocculently in S.B. with no opalescence. They produce compact colonies in S.P.A. with the exception of Type 20. In the india-ink preparation there is no suggestion of a halo surround the chains of cocci, the ink coming right up to the organisms. These generalizations have been summarized in Table 2.

TABLE 2.

*Correlation of Biological Characteristics of Haemolytic Streptococci (Human Source) with their Serological Grouping and Human Virulence.*

| Organism. | Serological Group. | Human virulence. | Appearance in S.B. | Appearance in S.P.A. | Capsule. | Halo in india-ink. | Colony form. |
|---|---|---|---|---|---|---|---|
| Capsulated | A | + | Diffuse growth with or without opalescence | Feathery semi-transparent colony | + | wide | "M" or conical or umbonate |
| Non-capsulated | A | + | Flocculent growth with opalescence | Compact opaque colony | 0 | narrow | "F" |
| Non-capsulated | not A | 0 | Flocculent growth without opalescence | Compact opaque colony | 0 | absent | conical, occasionally "F" |

*Exceptions Among Type Strains.*

Certain exceptions have been noted. The Type 4 and 12 cultures sent to us by Dr. Griffith do not grow as "F" colonies, nor do they show haloes in the india-ink preparation. However, Type 4 and Type 12 strains recently isolated by us behave like typical "F" variants belonging to Group A.

*Applications to the Study of Haemolytic Streptococci Recently Isolated from Human Sources.*

Using the same methods we have studied a large number of haemolytic streptococci isolated from the nasopharynx and from diverse human infections. With very few exceptions they have behaved in one or other of the three general ways described above. We feel confident that in the great majority of cases it is possible to detect Group A organisms by inoculating a tube of serum broth (S.B.) and a tube of serum peptone agar (S.P.A.). After incubation the growth characteristics are observed. These are interpreted in the following way:

1. Feathery semi-transparent colony in S.P.A. medium, with or without opalescence in S.B. medium = Group A.

2. Compact opaque colony in S.P.A. medium with opalescence in S.B. medium = Group A.

3. Compact opaque colony in S.P.A. medium, without opalescence in S.B. medium = not Group A.

We have met with two Group A strains which have behaved in an atypical manner. Both strains would have been mistaken for a Haemolytic Streptococcus not belonging to Group A on the evidence afforded by their growth in the S.B. and S.P.A. media. One was isolated from the nasopharynx of a healthy adult, the other from a chronic impetiginous lesion. On subsequent cultivation both strains threw off typical capsulated variants, so one must evidently look upon such strains as unstable. Neither of these strains was agglutinable by any of the Group A type sera.

We have not yet encountered a strain from a serious human infection which behaved in these media like a non-Group A organism. At the same time, we have little doubt that the non-Group A mucous variants which have been isolated from animal infections would behave in these two media like Group A mucous variants from human infections. Serious human infections can apparently be caused by non-Group A organisms, but only very occasionally. Unfortunately, we have not had available for study any such humanly virulent, non-Group A organisms.

We do not recommend either the demonstration of capsules or the india-ink preparation as routine tests because it is not easy to control the factors necessary for successful preparations.

## DISCUSSION.

The haemolytic streptococci from human sources have always been notoriously difficult organisms to work with in the laboratory owing to the biological and serological differences between the numerous strains. Thanks to the work mainly of Lancefield with her Group precipitin reaction, and of Griffith with his Type agglutination reaction in Group A, the classification of these organisms has now a sound

serological basis. The only drawback is the technical difficulty associated with these two reactions, making them somewhat unsuitable for use in a routine laboratory where a large amount of work has to be carried out in a limited time.

The work we present here can possibly be turned to account as a substitute for the precipitin test, since it is clear that the great majority of the potentially pathogenic Group A organisms behave in a characteristic way when grown in (1) serum broth and (2) serum peptone agar. It must, however, be recognized that occasionally an atypical Group A strain may not be detected by this method, and since these unstable strains throw off typical capsulated feathery variants, they must certainly be looked upon as potentially virulent.

We have attempted to throw a little light on the two new phenomena which have been described and which appear to be associated with virulence, namely, the opalescence in the serum broth medium and the feathery colony in the serum peptone agar medium.

*Opalescence.* This phenomenon appears to be due to the denaturation of serum protein by a ferment which is produced by every "F" variant belonging to Group A and a few "M" variants, when grown in a serum medium. It can be shown that the Seitz filtrate of a serum broth culture of an active strain will reproduce the effect when incubated with serum of the horse, rabbit, mouse or human being. (The sera of other animals have not been tested). The filtrate is inactivated by 0·4 p.c. potassium cyanide or by heating for 1 hour at 60° C.

We have investigated the possibility that the denaturation of the serum-protein might be an offensive weapon of the non-capsulated Group A strains, acting perhaps by inactivating complement and so interfering with phagocytosis. We could, however, find no definite evidence in favour of this theory.

*The Feathery Colony.* This type of colony in the serum peptone agar medium is definitely correlated with those organisms in which a capsular structure can be demonstrated in young culture, whereas the compact, opaque colony is seen in those organisms in which the young cocci are quite bare. The idea that the feathery type of colony is dependent on capsulation is strengthened when observations are made on the growth of virulent and avirulent pneumococci in this medium (Fig. 5). The virulent type pneumococci grow as elongated, feathery colonies, whereas the avirulent organisms appear as compact opaque colonies. On the other hand, such organisms as virulent non-motile typhoid bacilli or "smooth" dysentery bacilli, i.e. organisms in which no capsular structure can be demonstrated, also grow as elongated feathery colonies (Fig. 7). Incidentally the typhoid strain 0901, an organism known to have lost virulence, has a colony (Fig. 7) very like that of the avirulent pneumococcus.

Since the capsulated haemolytic streptococci and pneumococci and the virulent non-capsulated gram-negative bacilli all grow as feathery colonies, we suggest that the factor determining this type of colony is not necessarily the capsule

itself, but is possibly the charge on the surface of the actively multiplying organism. If this were the case, then one should be able to neutralize the charge with specific antiserum, and the compact opaque colony should develop if the normal serum in the medium is replaced by specific antiserum. With the pneumococcus this is true, since the Type 1 pneumococcus when grown in Type 1 antiserum peptone agar grows as a compact colony (Fig. 4) indistinguishable from the colony of the avirulent pneumococcus growing in normal serum peptone agar. With the streptococcus, however, the replacement of normal horse-serum by a strong type antiserum in the S.P.A. tube does not result in any change of the colony, i.e. it is still a feathery colony. Nevertheless, we do not think that this apparent contradiction invalidates the theory, because it can be shown that, in the agglutination of haemolytic streptococci, a strong Type antiserum is incapable of agglutinating a young culture of the Type organism grown in serum peptone. The capsulated haemolytic streptococcus loses its capsule as the culture ages, and it is only then that the organisms become agglutinable by the Type antiserum. Presumably the capsule of the young haemolytic streptococcus masks the deeper Type agglutinogen on which the Type antiserum acts. We have not been able to obtain an antiserum which will agglutinate a young culture of a capsulated haemolytic streptococcus when grown in a serum medium; one might suppose that it is only such an antiserum which could be expected to neutralize the charge on the surface of actively growing cocci and thus influence the form of colony in the weak agar medium.

The wide clear area surrounding the young capsulated cocci in india-ink preparations also suggests that these organisms, at this period of their growth, carry a strong negative charge which repels the ink particles. Further, we have studied india-ink preparations of young cultures of capsulated haemolytic streptococci and pneumococci grown in media containing normal serum or specific antiserum. The results have supported the hypothesis that the feathery colony is associated with a negative charge on the growing organism, for wherever a feathery colony was observed, a parallel ink preparation showed wide haloes, and wherever a compact colony was seen, the corresponding ink film showed the ink particles coming right up to the cocci. Thus, the capsulated haemolytic streptococcus showed a wide halo and a feathery colony in both normal serum and specific antiserum, but the Type 1 pneumococcus in normal serum showed a halo and a feathery colony, and in Type 1 antiserum had no halo and grew as a compact colony.

Although the charge theory seems a plausible explanation of the feathery colony in the case of the capsulated streptococci, it must be admitted that young non-capsulated streptococci belonging to Group A also show a definite halo in the india-ink preparations and yet grow as a compact colony in S.P.A. The halo is narrower with non-capsulated streptococci, and it is possible that the charge is here not strong enough to cause the growing chains to repel one another and lead to the feathery growth characteristic of the capsulated strains.

## CONCLUSIONS.

1. If haemolytic streptococci from human sources are grown in two media (a) serum broth and (b) serum peptone with just sufficient agar to suspend the developing colonies, the potentially pathogenic Group A (Lancefield) strains, with very few exceptions, can be differentiated by their growth characteristics from haemolytic streptococci belonging to the other serological Groups.

2. Capsulated Group A strains grow diffusely in the serum broth, and the colonies in the serum peptone agar have a feathery semi-transparent appearance.

3. Non-capsulated Group A strains grow flocculently in serum-broth with a marked opalescence in the supernatant fluid. The colonies in the serum peptone agar are compact and opaque.

4. Haemolytic streptococci from human sources, not belonging to Group A, grow flocculently in serum broth without opalescence in the supernatant fluid, and the colonies in serum peptone agar are compact and opaque.

5. Occasionally a Group A strain grows in an atypical manner in the two special media and may be mistaken for a haemolytic streptococcus not belonging to Group A. Such atypical strains are unstable, and throw off characteristic capsulated variants on cultivation.

6. The opalescence in serum broth produced by all the non-capsulated and a few of the capsulated Group A strains appears to be due to a ferment which denatures the serum protein.

7. It is suggested that the feathery colony in serum peptone agar which is characteristic of the capsulated Group A strains is due to the strong negative charge on the surface of the developing organism, resulting in the mutual repulsion of the chains of young cocci as they grow out into the medium. That this phenomenon is not necessarily associated with capsulation is indicated by the fact that non-motile virulent typhoid bacilli also develop feathery colonies.

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